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VOL. LII JANUARY–FEBRUARY, 1960 NO. 1

GROSS MORPHOLOGY OF THE PLASMODIUM AND ITS POSSIBLE SIGNIFICANCE IN THE RELATIONSHIPS AMONG THE MYXOMYCETES [1]

CONSTANTINE J. ALEXOPOULOS

(WITH 19 FIGURES)

The plasmodium, a multinucleate, acellular mass of protoplasm, usually nourished by engulfing solid particles of food, and capable of creeping over its substratum by an amoeboid movement, is the assimilative stage of the Myxomycetes.

Most active plasmodia encountered in nature belong to the Physarales. The plasmodia of such common Liceales as *Tubifera, Lycogala,* and *Reticularia,* and of most species of the Stemonitales are almost never seen before they are ready to fruit. In addition, because of difficulties encountered in growing on artificial media species of Myxomycetes other than Physarales, the plasmodium of the Physarales was, with a single exception, the only one which had been described in its various stages from zygote to the initiation of fruiting.

It was no accident, therefore, that the early workers, Cienkowski (**7**), de Bary (**4**) and Strasburger (**27**), who made the first detailed observations of the formation, development, and mode of life of the myxomycete plasmodium, based their descriptions almost exclusively on the plasmodia of such species as *Fuligo septica, Didymium difforme,*

[1] Based on experimental work supported by National Science Foundation Grant G-6382 for which I express deep appreciation.

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Physarum leucopus, and *Leocarpus fragilis.* The only species not belonging to the Physarales mentioned to any extent in the early developmental researches are *Perichaena corticalis* (*P. liceoides*) by Cienkowski and *Diachea leucopodia* (*D. elegans*) by de Bary.

De Bary (**4**), who summarized existing knowledge about the Myxomycetes in his Comparative Morphology, was very careful, time and again, to point out that what was known up to that time about the plasmodium of the Mycetozoa was based on the Physarales. Even though he believed that all plasmodia were essentially alike he did note some differences. Describing the plasmodia of *Didymium difforme* and *Physarum leucopus* as "usually much smaller" than those of other species of the Physarales (in which, however, he included *Diachea*) he continues "and this is the case with all the other forms which have been hitherto examined not belonging to the Physareae." Later he states that "the plasmodia of the Stemonitae, Trichiaceae, Ceratiae, and *Lycogala,* have in the main the same structure and power of movement as those of the Physareae. . . ." But after recording some observations on the plasmodia of the Cribrariae, *Dictydium, Arcyria punicea, Lycogala,* and *Stemonitis fusca,* he significantly concludes: "All the plasmodia last mentioned are inconspicuous bodies. . . . They live for the most part in the interior of rotten parts of plants, especially rotten wood, and are not visible to the naked eye till they come to the surface to form sporangia."

In 1892 Celakovsky (**6**) published what are probably the first important observations on the plasmodium of *Stemonitis.* He noted that the plasmodium of *S. fusca* (*S. dictyospora*) is unusual in the transparency of its strands and in the lack of differentiation of outer and inner plasma layers. Miller (**18**) also noticed the same transparency of the plasmodial strands in three unidentified species of *Stemonitis.* Shortly after Celakovsky's paper, Zukal (**30**) described the peculiar plasmodium of *Licea parasitica* as will be mentioned below.

In recent times, Thom and Raper (**28**) described a *Stemonitis* plasmodium as "consisting of very close networks of delicate strands of colorless protoplasm." Watanabe (**29**) and Nauss (**19**) have also pointed out that not all plasmodia are alike in their gross morphology, but unfortunately have not described major differences among them.

Inasmuch as most Myxomycetes which have been studied critically belong to the Physarales, and appear to have the same general type of plasmodium, it has been generally assumed that all myxomycete plasmodia have the same gross morphology, and, consequently, that of the Physarales has become the prototype of the myxomycete plasmodium

in the minds of most biologists. As might be expected, the concept of the myxomycetous plasmodium is even narrower for the ever increasing group of investigators who are not interested in the Myxomycetes *per se*, but only for their usefulness as tools in cytological, biochemical, and biophysical research. For them, there is only one plasmodium—that of *Physarum polycephalum*. Nevertheless, there appear to be at least three major types of plasmodia in the Myxomycetes which are readily recognizable if they are properly observed at the right stage of development.

In 1893 Zukal (**30**) studying *Licea parasitica* (*Hymenobolina parasitica*) succeeded in culturing the organism on willow bark and described its plasmodium as minute, nearly immobile, and devoid of either vein-like structures or rhythmic, reversible protoplasmic streaming. Chiefly for this reason Lister (**12**) termed this species "exceptional among the Mycetozoa . . ." and so it remained until 1959 when we succeeded in growing *Echinostelium minutum* in agar culture (**1**).

It has long been suspected that the plasmodium of *Echinostelium minutum* does not grow beyond microscopic size and that it gives rise to but a single sporangium. Peterson (**20**) was the first to record this belief based on his observations of bark cultures. When this organism was finally cultivated on artificial media (**1**), some interesting facts concerning it were revealed.

Whether the plasmodium of *E. minutum* begins as a zygote has not been elucidated up to this time. I have reason to believe that the species may be apogamic, but this remains to be proved. Be that as it may, the plasmodia of this species are recognizable first by their size when they attain a diameter 20–30 μ. At this stage the plasmodium appears to be a large myxamoeba. It has a flattened aspect, a more or less round outline, and at least one contractile vacuole. It is colorless; it extends and withdraws many very short pseudopodia along its periphery; it migrates very slowly over the surface of the agar; and it exhibits almost imperceptibly slow streaming without a discernible pattern. At this stage the plasmodium of *Echinostelium minutum* is difficult to distinguish from equally young plasmodia of many other Myxomycetes which may be grown on artificial media, and on which, therefore, observations may be made readily. (FIGS. 1–3, 14.)

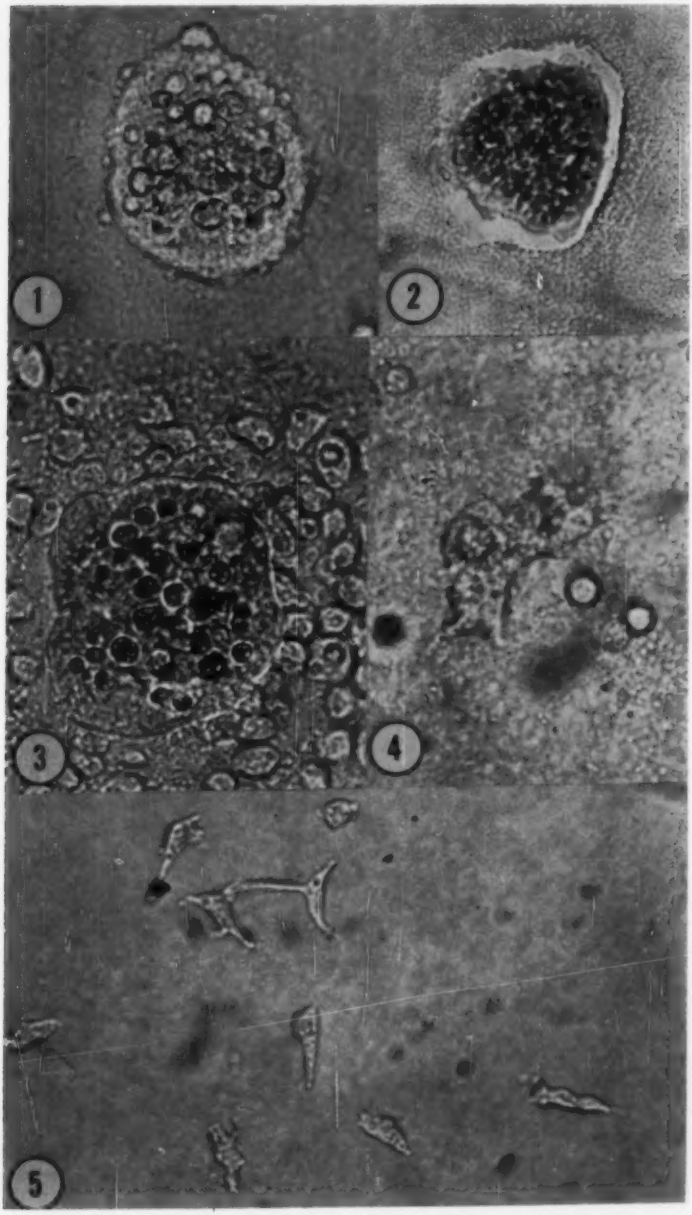
But, whereas the plasmodia of most other Myxomycetes which have been studied soon develop further and assume a different aspect from that of their initial phase, the plasmodium of *Echinostelium minutum*, in common with that of *Licea parasitica*, changes but slightly from its original form except in size. The mature plasmodium just before it fruits is still more or less homogeneous in structure; the ground sub-

stance is granular; vacuoles are conspicuously present, some contractile, some food vacuoles. The plasmodium may remain more or less round, or it may at times be irregular in outline, but it has no differentiated, advancing, fan-shaped region; it has no veins; it exhibits no reticulation whatsoever at any time (FIG. 1); and, most interesting of all, no rapid rhythmically reversible streaming. The streaming continues to be very slow and conspicuously irregular.

In these respects, the mature plasmodium—using this designation to indicate a stage just before fruiting—strikingly resembles the plasmodia of the Physarales at a stage immediately following the zygote (FIG. 3), and can be said, therefore, to retain juvenile characteristics throughout its existence. A new species of *Echinostelium*, which we grew in culture recently, has the same type of plasmodium. Cultures which were started from spores of *E. elachiston* also produced similar plasmodia (FIG. 2). Recently Sister Mary Annunciata McManus has found (personal communication) that the plasmodium of *Clastoderma debaryanum* exhibits the same characteristics. Because it appears to me that this is the most primitive type of plasmodium known in the Myxomycetes, I am designating it as a protoplasmodium (πρῶτος = first) to distinguish it from the other types which are discussed here and which I believe to be more advanced.

The plasmodia of various species of *Stemonitis* and of *Comatricha*, such as *C. typhoides*, and *C. irregularis*, grow in nature within the woody substratum on which they generally fruit, but are not encountered until they come to the surface just before sporangial formation. Thus the plasmodia of these two genera are not usually seen as protoplasmic networks, such as those formed by the familiar *Physarum polycephalum* or *Physarella oblonga*, which spread over the substratum long before fruiting, but only as protoplasmic clumps which soon begin to assume the shape of the fructifications which they are destined to produce. Although Celakovsky, Miller, and Thom and Raper had pointed out some important characters of the growing plasmodium of *Stemonitis*, it was not until 1959 (2), when we were able to follow the development of the plasmodium of *Stemonitis flavogenita* in agar culture from its earliest stages to the time of fruiting, that the full distinction between the "stemonitoid" plasmodium and that of the Physarales was brought out,

FIGS. 1–4. Plasmodia of four species of Myxomycetes at approximately the same stage of development on Difco corn meal agar. FIG. 1. *Echinostelium minutum*. FIG. 2. *E. elachiston*. FIG. 3. *Fuligo cinerea*. FIG. 4. *Stemonitis fusca*. Note elongated pseudopodia, particularly at the lower portion. FIG. 5. Myxamoebae of *S. fusca*, several with elongated pseudopodia. All × 570.



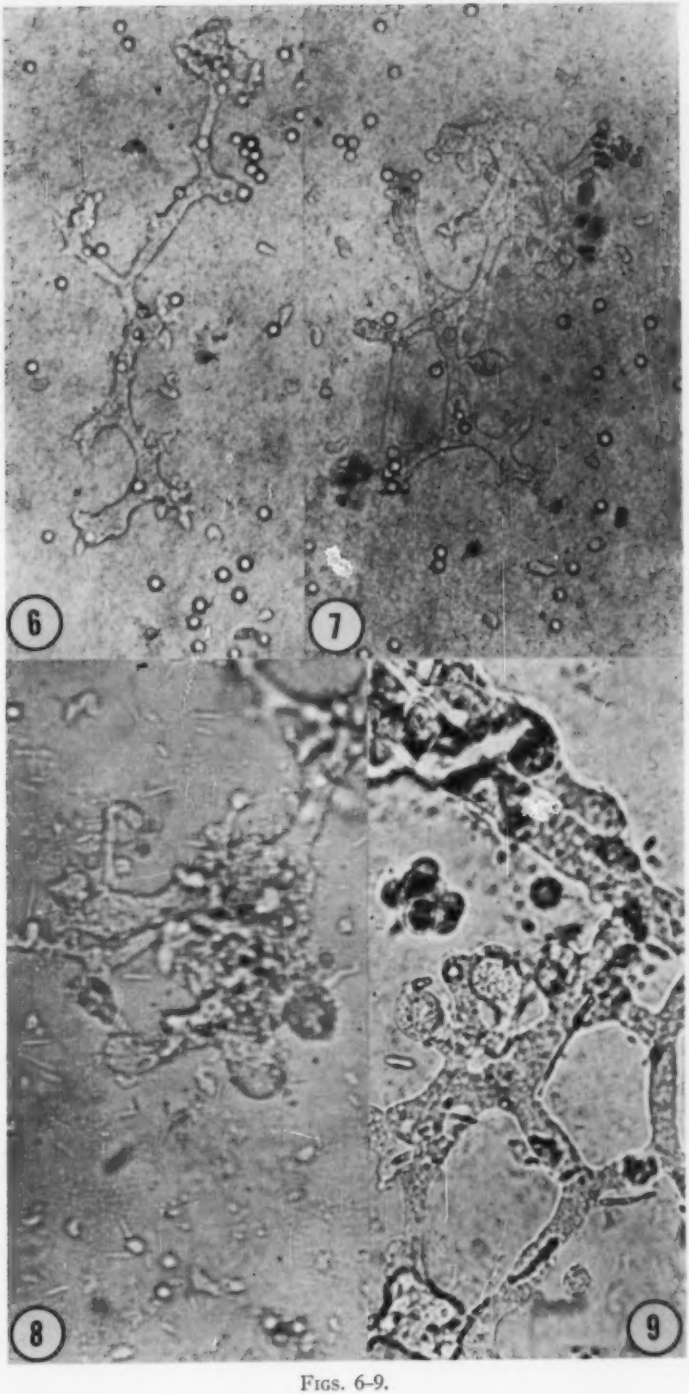
FIGS. 1–5.

confirming and expanding the observations of earlier investigators. Further studies from our laboratory (3) and the work of McManus and Richmond (13) on *Stemonitis fusca* leave little doubt that the "stemonitoid" plasmodium represents a distinct type.

More often than not an agar culture of *Stemonitis flavogenita* or *S. fusca* may be recognized from the time the swarm cells lose their flagella and become myxamoebae, for the myxamoebae of these two species at least have a tendency to be more irregular in outline, some appearing almost asteroid, and often extending a long pointed pseudopodium in one direction (FIG. 5). It is not rare in such cultures to see pairs of myxamoebae with their long, pointed pseudopodia in contact, possibly copulating. It is nevertheless true that very irregular myxamoebae are often seen in cultures of physaraceous species so that this is not an infallible criterion. The first recognizable stage of a "stemonitoid" plasmodium may be a more or less rounded mass of protoplasm. Such a structure is not much different from a very early stage of the plasmodium of any of the Physarales which have been grown in culture. Often, however, in contrast to the plasmodia of both *Echinostelium* and many of the Physarales, that of *Stemonitis* is already elongated and has a tendency to extend pointed pseudopodia (FIG. 4) which soon give it a very irregular outline. At this stage it may send out a long, hypha-like strand which may lead to another highly irregular mass, or it may produce a few branches (FIG. 6) which will eventually anastomose and begin to form a network (FIG. 7). The plasmodium, which may now be 100 μ or more in length, is greatly flattened and highly transparent. The hypha-like strands have parallel sides, and are about 5–10 μ in diameter. The protoplasm is confined by a very thin membrane, inside of which the entire protoplast exhibits a rhythmical, reversible streaming which sometimes is so slow as to be almost imperceptible, at other times quite rapid.

Even after a considerable network has been formed, the "stemonitoid" plasmodium may have no definite margin and no differentiated advancing fan. Strands protrude in every direction from different parts of the net, sometimes ending abruptly, sometimes terminating in vesiculose, irregular swellings (FIGS. 8–9). The latter often spread out, giving the impression of a bursting bubble allowing the jelly-like contents to flow out rapidly and form a puddle. Such puddles from adjacent vesicles may coalesce sometimes, and form small protoplasmic sheets some-

FIGS. 6–7. *Stemonitis fusca*. Young plasmodia on Difco corn meal agar. FIGS. 8–9. *S. herbatica*. Vesiculose terminal swellings of plasmodial branches. All × 570.



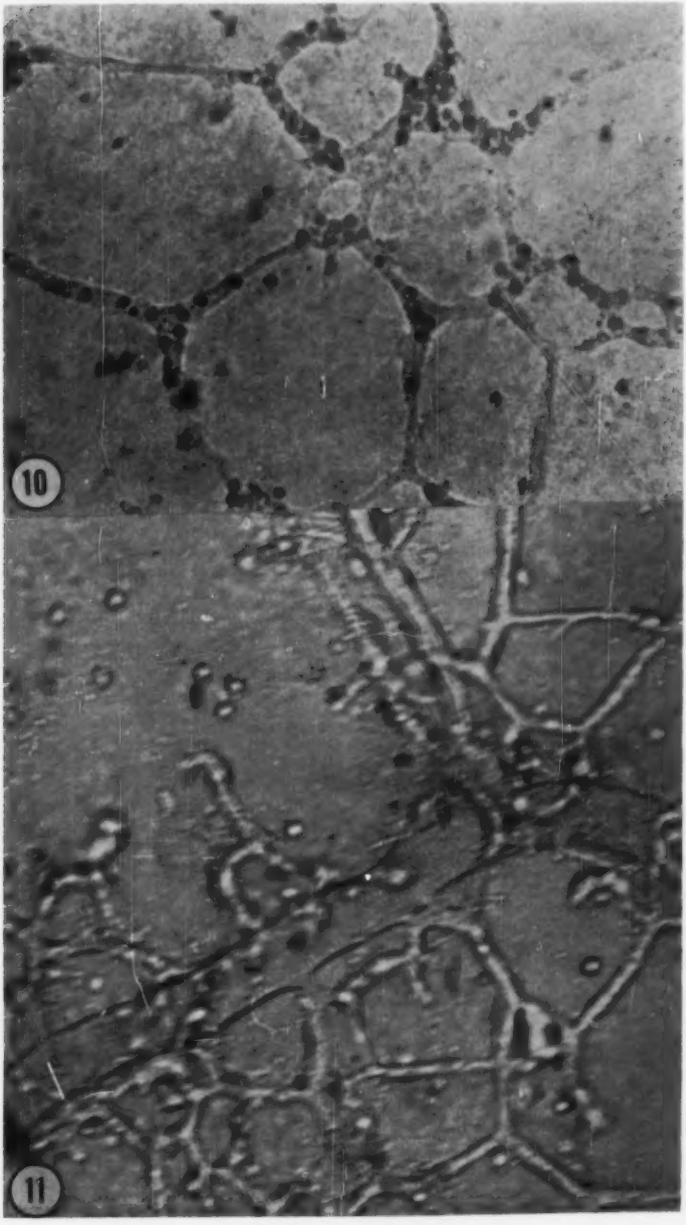
FIGS. 6–9.

what resembling small fans of a physaroid plasmodium. From this form the plasmodium grows rapidly and may assume a variety of shapes. Quite often strands will grow out for a considerable distance without branching. Eventually branches are produced forming a large and often symmetrical net-work (FIG. 10) with large meshes 100–200 μ in diameter or more. These strands are nearly isodiametric, and their resemblance to hyphae is so striking that the observer cannot help but be strongly influenced in favor of Martin's arguments for the fungal affinities of the Myxomycetes (**17**). At other times, these hypha-like strands will produce many branches at short intervals which may re-branch and anastomose, forming small nets with meshes 20–80 μ in diameter, or which may end abruptly. At still other times the main strand of a plasmodium may be quite large, reaching a diameter of 18–35 μ or more. Such a strand is often surrounded by a thin net-work of fine strands which issue as branches from it (FIG. 11). The protoplast of a thick strand of this type is often, but not always, differentiated into a non-motile outer region and a streaming central core.

As the plasmodium grows older, it forms one or more fans with definite margins and its general shape begins to resemble somewhat the well-known physaraceous type. Nevertheless, even at an advanced age and after a considerable size has been reached, the "stemonitoid" plasmodia are so flat and transparent that they are all but invisible to the unaided eye and are discernible only with great difficulty (FIG. 12) under a dissecting microscope by reflected light. Indeed, it appears probable that the seeming failure to grow *Stemonitis* in agar culture previous to 1959 was not so much a failure to obtain plasmodia from spores, but rather a failure to recognize that plasmodia had formed, which led to a subsequent discarding of perfectly good cultures. This "invisibility" of the immature stemonitoid plasmodium is one of its chief characteristics and prompts me to designate this type as an aphanoplasmodium (*ἀφανής* = invisible). Only when the plasmodium is about to fruit do its strands thicken and become coralloid (FIG. 13). The plasmodium is then readily visible but still has a decidedly different aspect from that of most Physarales.

The best known plasmodial type is the physaraceous, exhibited by all species of Physarales which have been studied. As has been stated in the introduction to this paper, it has come to be considered the prototype of the myxomycete assimilative stage.

FIGS. 10–11. *Stemonitis fusca*. FIG. 10. Plasmodial network of hypha-like strands. FIG. 11. Large strand surrounded by network of finer strands which issue as branches from it. Both × 570.



FIGS. 10–11.

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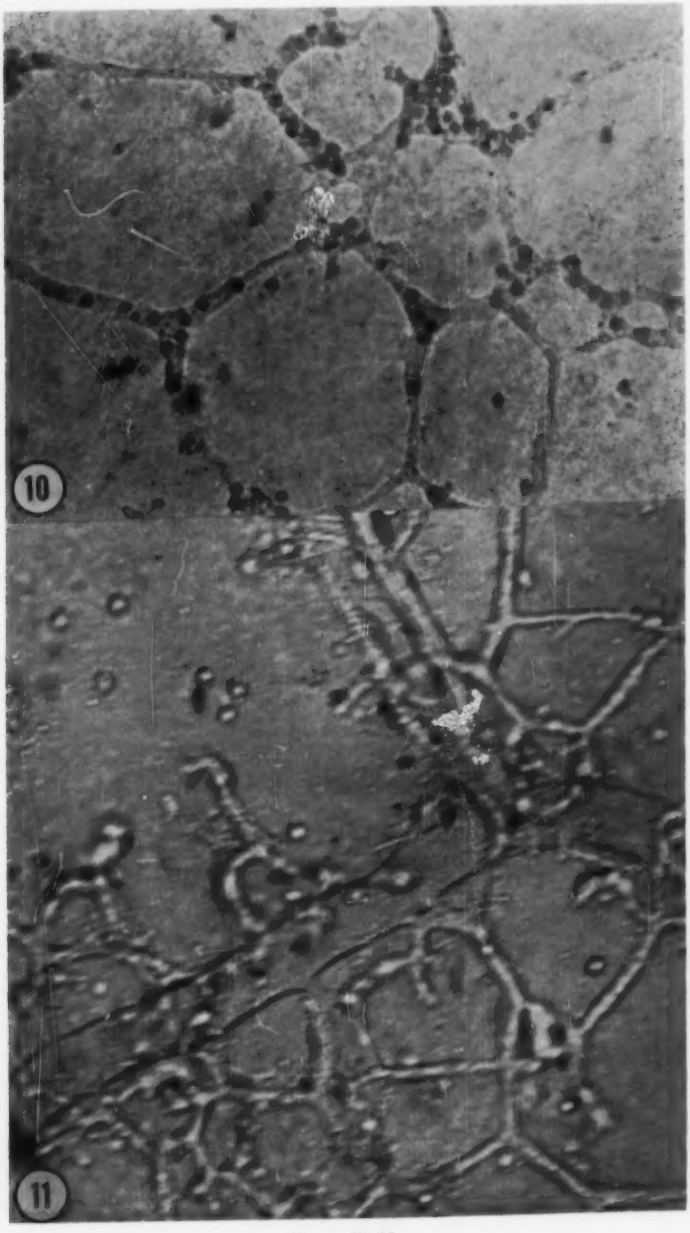


FIGS. 10–11.

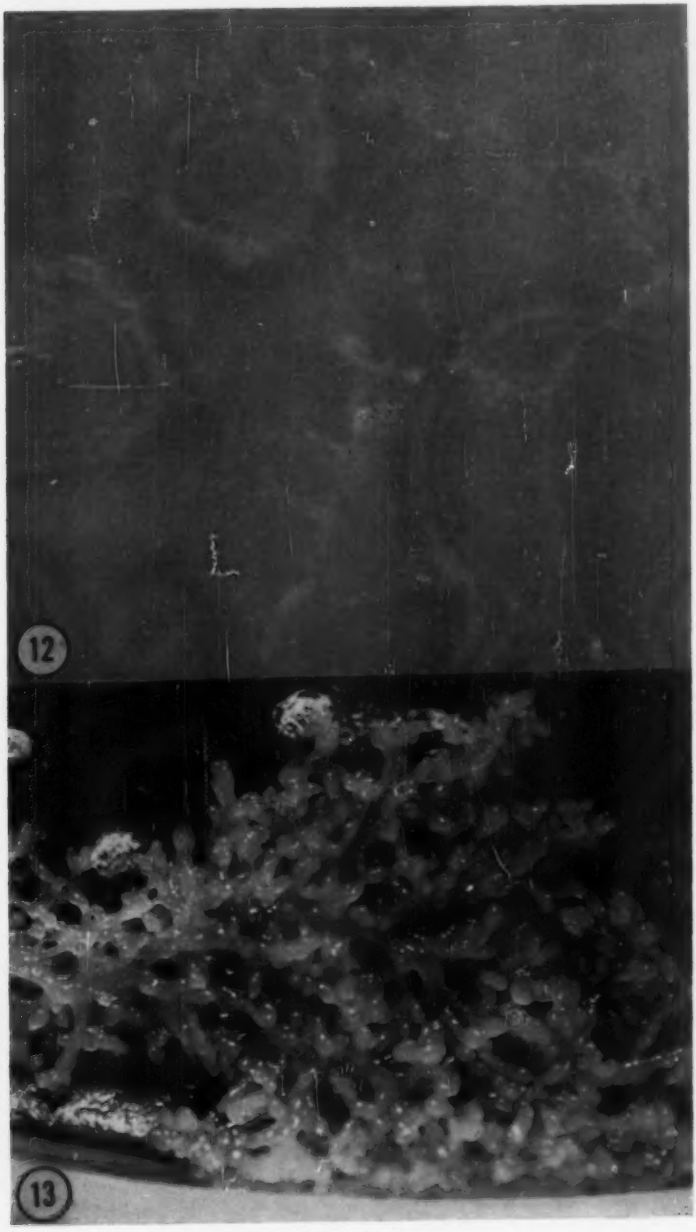
Many studies (**7, 10, 11, 22, 24**) indicate that the plasmodium of the Physarales begins as a zygote. With successive nuclear divisions the zygote soon grows into a minute plasmodium. By the time it reaches about 30–40 μ in diameter streaming becomes detectable with a magnification of $\times$ 400. Streaming is confined to the central part of the plasmodium. It is very slow and irregular. Stewart and Stewart (**26**) state that in *Physarum polycephalum,* such very small plasmodia show no definite channel structure. Reversal-like changes occur occasionally, but not regularly in such plasmodia. My own observations indicate that the situation is similar in at least ten other species of Physarales I have examined. At this stage (FIGS. 3, 14) this type of plasmodium is hardly distinguishable from a protoplasmodium at the same stage of development. Soon, however, the physaraceous type of plasmodium becomes elongated and develops a definite channel in which the protoplasm begins to stream back and forth more or less rhythmically. One end of the elongated structure soon becomes wider. Polarity has now been established and the plasmodium moves in the direction of its wider portion (FIG. 15). The anterior portion now begins to take the shape of a fan (FIG. 16). The differentiation of the plasmodial protoplast into a streaming and a non-streaming portion is very definite, the non-streaming, jellified outer region of the physaraceous plasmodium being very prominent at all times.

As this plasmodium grows, the fan thickens and develops into a sheet of protoplasm (FIG. 18) in which several streaming channels originate, the most prominent ones being oriented from front to back, with shorter channels oriented crosswise or diagonally to the main veins. Farther back the plasmodium becomes reticulated, but the strands which form the net are not regular and hypha-like as they are in the aphanoplasmodium, but irregularly wide. In a small plasmodium of *Physarella oblonga* (**3**) which measured no more than 2 mm in length, the main strands which formed the reticulum varied from 30 to 60 μ in width. This is 3 to 5 times the thickness of the comparable hypha-like strands of an aphanoplasmodium at the same stage of development.

Camp (**5**) described the structure of the vigorously growing plasmodium of *Physarum polycephalum* as follows:

". . . the plasmodia spread over the surface of the substratum in

FIGS. 12–13. *Stemonitis fusca.* FIG. 12. Large plasmodium on Difco corn meal agar, photographed by reflected light through one 15 $\times$ eyepiece and $\times$ 1 objective of an AO stereoscopic microscope. Compare with FIG. 19 of a plasmodium of *Physarella oblonga* photographed under the same conditions. $\times$ 8. FIG. 13. *Stemonitis* sp. Plasmodium just before fruiting, showing coralloid structure. $\times$ 5.



FIGS. 12–13.

the form of perforated sheets and anastomosing networks of vein-like branches. The advancing or anterior margin, . . . very frequently presents an irregular or lobed appearance. . . .

"In plasmodia whose advancing margin is more or less continuous or smooth . . . the protoplasmic material back of the margin is very frequently distributed in a continuous sheet. . . . The displacement of protoplasm is most conspicuous along stream-like or channel-like courses which are thicker and less transparent than adjacent portions of the sheet. It must be emphasized that these courses are not sharply defined or limited. On the contrary the impression gained . . . is that the active protoplasmic streaming is more or less limited to material located in the center of the streams and the surrounding protoplasm tends to be pulled along. . . .

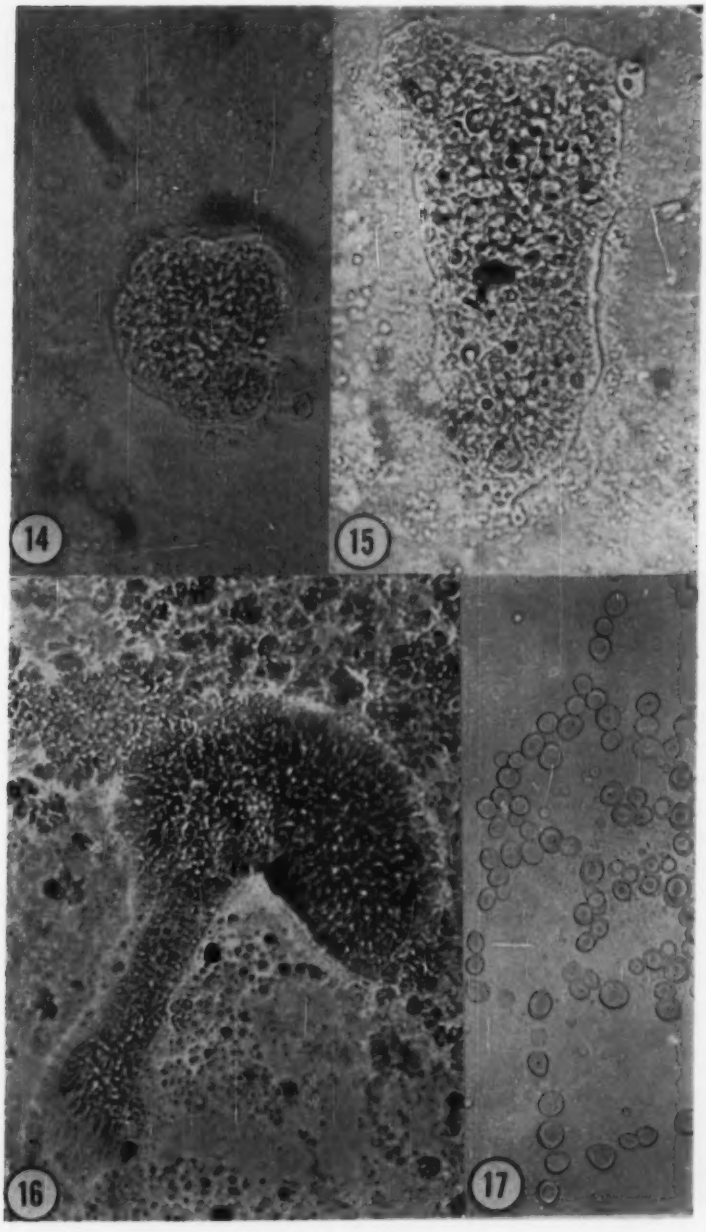
". . . the reticula may develop in two ways, namely, by branching at the margin of the plasmodium followed by the anastomosis of branches which happen to come in contact, and by the development of perforations in continuous plasmodial sheets followed by the displacement of protoplasm away from these perforations and into vein-like strands."

Exactly how the reticulated pattern of the stemonitoid aphanoplasmodium develops has not as yet been determined, but the absence of prominent sheet-like fans until a late stage of development indicates strongly that the early reticulation pattern develops solely by the anastomosis of the hypha-like strands.

Another characteristic of the physaraceous plasmodium, certainly correlated with the structure described above, is its obvious three dimensional appearance, in contrast to the flatness of the aphanoplasmodium. The physaraceous plasmodium, visible to the unaided eye even when it is no larger than 1 mm in length, is very conspicuous at a later stage when observed by reflected light under a binocular dissecting microscope (FIG. 19) at a magnification of × 15. The protoplasm here appears to have a greater density than in the stemonitaceous plasmodium. Because of the ease with which this third plasmodial type may be observed, it appears suitable to designate it as a phaneroplasmodium (*φανερός* = visible).

My observations to date indicate that aphanoplasmodia and phaneroplasmodia differ in physiology as well as morphology. Aphanoplasmodia never, in my experience, develop in culture if the agar surface is per-

FIGS. 14–16. *Physarella oblonga*, white form. Plasmodia on Difco corn meal agar at successive stages of development. FIG. 17. *Stemonitis fusca*. Cysts formed on dry agar surface by a sclerotized plasmodium. FIGS. 14, 15, 17, × 570. FIG. 16, × 1350.



FIGS. 14–17.

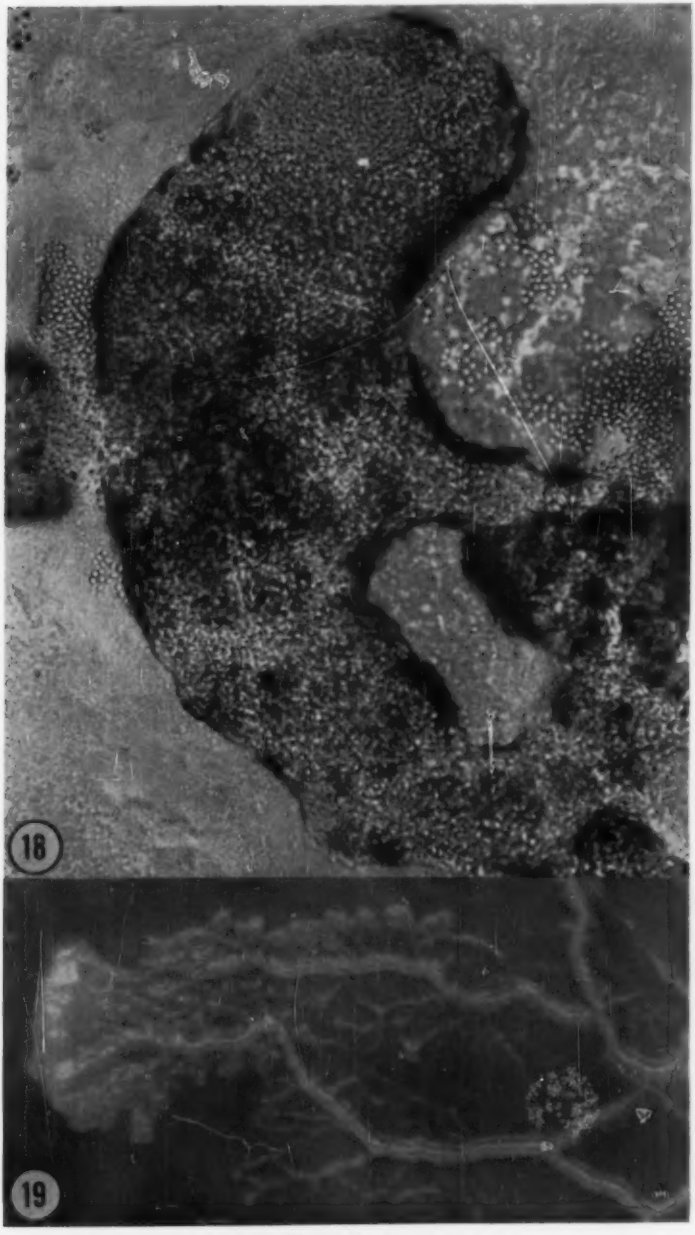
mitted to dry. Even after they are well formed they will generally sclerotize, breaking up into a large number of cysts (FIG. 17) if they are not covered with water at all times. These cysts will often reform into a plasmodium if water is added to the culture. Only at a very late stage in their development, as they approach the fruiting phase, will aphanoplasmodia thrive on a dry agar surface. The formation of phaneroplasmodia, on the other hand, appears to be usually favored by dryer conditions, although many species of Physarales will develop plasmodia under water.

The characteristics of the three plasmodial types I have just described are rather clear cut. In a laboratory such as ours in which several people are working with a large number of myxomycetous species, culture contaminants are as likely to be Myxomycetes as other fungi. On a number of occasions when spores of myxomycete species other than *Stemonitis*, or *Comatricha* had been planted and "stemonitoid" plasmodia developed, we predicted that such plasmodia represented contaminants and belonged to one of the Stemonitaceae. When the cultures fruited the prediction always proved to be accurate. Also on a few occasions when *Echinostelium* cultures became contaminated with what eventually proved to be *Didymium iridis*, the identity of the plasmodia as "physaraceous" was easily discernible while the plasmodia were still of microscopic size.

Whereas I believe that the three plasmodial types I have described represent distinct entities, I am not at all certain that, as our knowledge increases, the plasmodia of all the Myxomycetes will fall within these three taxonomic pigeon holes. As a matter of fact, I am already suspecting that we shall find intermediate forms, for it appears that some of the Trichiales, mention of which has been studiously avoided up to now in this discussion, may be the first to rebel against such a clear cut classification of plasmodial types.

In May 1958 a culture in which spores of *Physarella oblonga* had been planted yielded 3 sporangia of . . . *Arcyria cinerea!* This, to my knowledge, is the first time that any member of the genus *Arcyria* has ever fruited in agar culture. Spores from the well-formed fruiting bodies were planted on May 11 on corn meal agar, germinated in less than 24 hours, and yielded plasmodia in a few days. The following brief notes were recorded in my notebook on May 20, 1958. "Several plasmodia visible under the low power of the stereoscopic microscope. Some are

FIGS. 18–19. Plasmodia of *Physarella oblonga*, white form. FIG. 18. First well-developed fan. × 420. FIG. 19. Small but well-developed plasmodium photographed by reflected light. Compare with FIG. 12. × 8.



FIGS. 18–19.

quite large. Structure intermediate between Stemonitales and Physarales. Fans of Physarales with slow streaming and indefinite 'cortex' of Stemonitales." By June 19 the organism had completed its life cycle, having produced several typical sporangia on the agar and on the sides of the Petri dish.

The plasmodium of *Arcyria cinerea* in culture is an aphanoplasmodium in the sense that it is not conspicuous until it attains a considerable size; in that, in its early stages, it exhibits a large number of vesiculose projections such as those described for *Stemonitis;* and in that it develops only under water. On the other hand, it resembles the phaneroplasmodium of the Physarales in the formation of typical advancing fans at a rather early stage in its development, and in that its protoplasm appears to be much more granular than that of the stemonitaceous plasmodium.

Whether the plasmodium of *Arcyria cinerea* represents a fourth distinct type is too early to determine. Obviously a much more detailed study is necessary before any but the most tentative conclusions may be drawn concerning that species. *Hemitrichia vesparium* is the only other species in the Trichiales which has been grown in culture from spores. *Hemitrichia vesparium* was induced to complete its life cycle in culture from spore to spore by W. D. Gray (**9**) in 1938. Unfortunately, Gray did not describe the early stages of the plasmodium. Our attempts to grow this species in culture have failed up to now.

Having established the existence of at least 3 major types of plasmodia in the Myxomycetes, let us now examine the possible significance of these types as an indication of relationship of the species which exhibit them.

The classification of the Myxomycetes has undergone a considerable evolution since the time of Linnaeus whose writings are the starting point for the nomenclature of these organisms. Many systems have been devised, from that of Rostafinski (**23**) in the first reasonably complete monograph of the group, to the latest treatments by Martin (**16**, **17**) which represent the most modern thinking on the taxonomy of the Myxomycetes. All of these systems are based strictly on the structure of the spores and the spore-bearing bodies and completely neglect the plasmodium. Indeed, in several of the species no one seems to have ever seen such a structure, its existence being conjectured from the appearance of the sporophores which are obviously myxomycetous. The reasons for the neglect of the plasmodium in taxonomic thinking are obvious after the discussion of plasmodial structure which formed the main body of this paper. Our knowledge of plasmodial structure was so limited until

recently that the usefulness of the assimilative stage of the Myxomycetes as a taxonomic criterion was in great doubt. Martin (**15**) wrote in 1940: "Genera, families and orders are based on characters of widely varying significance, as a result of which closely related species are often placed in different genera or families. At the present time, it seems unlikely that study of the plasmodia will throw much light on this subject, although that, of course is not impossible. . . ."

If we group the myxomycete species whose plasmodia have been studied critically from an early stage in their formation to maturity or near maturity, under the 3 major types which have been discussed in this paper, we have:

PROTOPLASMODIUM	APHANOPLASMODIUM
Echinostelium minutum	*Stemonitis flavogenita*
Echinostelium cribrarioides[2]	*Stemonitis fusca*
Echinostelium elachiston (?)[3]	*Stemonitis herbatica*[4]
Clastoderma debaryanum	*Comatricha irregularis* (?)
Licea parasitica	*Comatricha typhoides* (?)

PHANEROPLASMODIUM

Fuligo cinerea	*Physarum oblatum*
Fuligo septica	*Physarum polycephalum*
Physarella oblonga	*Physarum pusillum*
Physarum compressum	*Physarum tenerum*
Physarum didermoides	*Didymium difforme*
Physarum gyrosum	*Didymium iridis*
Physarum leucophaeum	*Didymium squamulosum*
Physarum nucleatum	

For the purpose of this discussion, let us assume that the plasmodia in the above list which have not fruited belong indeed to the organisms named and were not contaminants. Particularly if that is so, certain interesting correlations may be detected. Five species in 2 genera of the Stemonitaceae produce an aphanoplasmodium. No species outside this family has been found to possess this plasmodial type. Sixteen species in 4 genera and in both families of the Physarales produce a phaneroplasmodium. No species outside this order has been found to possess this plasmodial type, nor has any species in this order been

[2] New species. Description in press, Amer. Midl. Nat.

[3] Question mark denotes that plasmodia of the types indicated developed but did not fruit, in cultures in which spores of the species named had been planted. Their identity must, therefore, remain in doubt.

[4] Tentative identification by G. W. Martin of a species of *Stemonitis* collected in Texas and sent to the writer by Dr. James Maniotis.

found up to now to have either of the other two types of plasmodium. The seeming correlation between plasmodial type and taxonomic position breaks down, however, when the protoplasmodial group is examined. All 3 known species of *Echinostelium* possess a protoplasmodium, but *Clastoderma debaryanum*, one of the Stemonitales and *Licea parasitica* in the Liceales also produce the same type of plasmodium. This, in a way, is not surprising. All five of these species produce very minute sporangia. It is possible that future studies will show that many, if not all, of the minute species produce but a single sporangium from each plasmodium which may reasonably be expected to be a protoplasmodium. No one, up to now, has succeeded in growing such species as *Comatricha fimbriata, Comatricha laxa, Cribraria minutissima, Licea kleistobolus, Licea operculata*, etc., in artificial culture. It appears probable that the plasmodia of many such species will belong to the protoplasmodial group.

Whether other Stemonitaceae produce aphanoplasmodia remains to be discovered. It may reasonably be expected that most species of *Stemonitis* will fall into the same pattern. If *Comatricha typhoides* indeed possesses an aphanoplasmodium, most Lamprodermas may be expected to conform. *Diachea* may depart from this scheme. The extensive plasmodium of *Diachea leucopodia* has been known since the time it spread in Fries' hat (**14**). No one appears to have followed its development. Ross (**22**) mentioned this species in his recent study but did not describe the plasmodium. It may be that this plasmodium shows strong affinities to the phaneroplasmodium of the Physarales. This being the only genus of the Stemonitales in which lime is present, its physiology approaches that of the Physarales and *Diachea* may represent a link between the two orders.

What about the Trichiales and Liceales? Too little is known about them to permit even conjecture. *Arcyria cinerea* and *Hemitrichia vesparium* are the only two Trichiales which have been induced to complete their life cycles in culture (**3**, **9**). As stated earlier, *Arcyria cinerea* appears to have an assimilative stage intermediate between the aphanoplasmodium of the Stemonitaceae and the phaneroplasmodium of the Physarales, but showing greater resemblance to the former. The plasmodium of *Perichaena corticalis* was obtained in culture by Cienkowski (**7**), but no conclusion can of course be drawn from his remarks with reference to the types of plasmodia being discussed here. His figures, nevertheless, may provide some interesting clues. Figure 4 in Plate XIX very much resembles an early stage of an aphanoplasmodium (FIG. 6), and his Figure 1 in the same plate is greatly reminiscent of a young plasmodium of *Arcyria cinerea* in culture, which, as was stated

earlier, exhibits characteristics of both aphanoplasmodia and phaneroplasmodia. It will not be surprising to find many of the Trichiales occupying an intermediate position between the Stemonitales and the Physarales in the morphology of their plasmodium.

The plasmodium of *Licea variabilis* (*Licea flexuosa*) has been maintained in culture for a long time by Sobels (**25**). Her photographs give the impression that the well developed plasmodium is very much like those of the Physarales, but she does not describe the early stages.

It is of course too early to determine how close a correlation exists between the plasmodial types and the sporophore types on which our present system of classification is based, but a beginning has been made and some trends are appearing. Many more species must be investigated critically, but it appears now that a study of the plasmodium and of the mode of its evolution into the spore bearing stage (**21**) may be of considerable importance in determining relationships among the Myxomycetes which our taxonomic system should eventually attempt to reflect.

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LITERATURE CITED

1. **Alexopoulos, C. J.** Morphology and laboratory cultivation of *Echinostelium minutum* de Bary. Am. Jour. Bot. **47**: 37–43. 1960.
2. ——. The laboratory cultivation of *Stemonitis*. Am. Jour. Bot. **46**: 140–142. 1959.
3. ——. Unpublished research.
4. **Bary, A. de.** Comparative morphology and biology of the fungi, Mycetozoa, and bacteria. (Engl. transl.) XVIII + 525 pp. Illus. Clarendon Press, Oxford. 1887.
5. **Camp, W. G.** The structure and activities of the myxomycete plasmodia. Bull. Torrey Bot. Club **64**: 307–335. 1937.
6. **Celakovsky, L.** Ueber die Aufnahme lebender und todter verdaulicher Körper in die Plasmodien der Myxomyceten. Flora **76**: 182–244. 1892.
7. **Cienkowski, L.** Das Plasmodium. Jahrb. wiss. Bot. **3**: 400–441. 1863.
8. **Constantineanu, J. C.** Über die Entwickelungsbedingungen der Myxomyceten. Ann. Mycol. **4**: 495–540. 1906.
9. **Gray, W. D.** The effect of light on the fruiting of Myxomycetes. Am. Jour. Bot. **25**: 511–522. 1938.
10. **Howard, F. L.** The life history of *Physarum polycephalum*. Am. Jour. Bot. **18**: 116–133. 1931.
11. **Jahn, E.** Myxomycetenstudien VIII. Der Sexualakt. Ber. Deutch. Bot. Gesellsch. **29**: 231–247. 1911.
12. **Lister, A. L.** A monograph of the Mycetozoa. Ed. 3. Revised by Gulielma Lister. XXXIII + 295 pp. Illus. British Museum, London. 1925.

13. **MacManus, *Sister* Mary Annuciata, and *Sister* M. Vianney Richmond.** Spore to spore culture on agar of *Stemonitis fusca*. Am. Midl. Nat. **65**: 246. 1961.
14. **Martin, G. W.** The contribution of de Bary to our knowledge of Myxomycetes. Proc. Iowa Acad. Sci. **65**: 122–127. 1958.
15. —— The Myxomycetes. Bot. Rev. **6**: 356–388. 1940.
16. ——. The Myxomycetes. N. Am. Flora **1**. pt. 1: 1–190. N. Y. Bot. Gard., New York. 1949.
17. ——. The systematic position of the Myxomycetes. Paper presented before Section G3 of the AAAS, December 29, 1959. Mycologia. (In Press.)
18. **Miller, C. A.** The aseptic cultivation of Mycetozoa. Quart. Jour. Micr. Sci. **41**: 43–71. 1898.
19. **Nauss, R. N.** My garden of slime molds. Jour. N. Y. Bot. Gard. **48**: 101–109. 1947.
20. **Peterson, J. E.** Myxomycetes developed on bark of living trees in moist chamber culture. Master's thesis. Mich. State Coll. East Lansing. 1952.
21. **Ross, I. K.** Capillitial formation in the Stemonitaceae. Mycologia **49**: 809–819. 1957.
22. ——. Syngamy and plasmodium formation in the Myxogastres. Am. Jour. Bot. **44**: 843–850. 1957.
23. **Rostafinski, J. T.** Slúzowce (Mycetozoa). Monographia and supplement. 432 pp. Illus. Kórnickiéj. Paryz. 1874–75.
24. **Skupienski, F. X.** Recherches sur le cycle évolutif de certains Myxomycètes. Paris. 1922.
25. **Sobels, J. C.** Nutrition de quelques Myxomycètes en cultures pures et associées et leurs propriétés antibiotiques. 136 pp. Illus. Gouda, Netherlands. 1950.
26. **Stewart, P. A., and Babette T. Stewart.** Protoplasmic movement in slime mold plasmodia: The diffusion drag force hypothesis. Exp. Cell Res. **17**: 44–58. 1959.
27. **Strasburger, E.** Zur Entwickelungsgeschichte der Sporangien von *Trichia fallax*. Bot. Zeit. **42**: 305–316; 321–326. 1884.
28. **Thom, C., and K. B. Raper.** Myxamoebae in soil and decomposing crop residues. Jour. Wash. Acad. Sci. **20**: 362–370. 1930.
29. **Watanabe, A.** On the plasmodium of the Myxomycetes (trans. title). Kagaku **10**: 418–421. 1932.
30. **Zukal, H.** Über zwei neue Myxomyceten. Oester. Bot. Zeitschr. **43**: 73–77; 133–137. 1893.

SYNCHYTRIUM TEXANUM SP. NOV.[1]

JOHN S. KARLING

(WITH 13 FIGURES)

Synchytrium texanum was discovered on *Plantago rhodosperma* near Austin, Texas in April 1958 in a low sparsely wooded area which is flooded occasionally for brief periods by the spring rains. This area is about one-half acre in extent; in 1958 it was covered by an almost pure stand of *P. rhodosperma*. Nearly all these plants were densely infected by *S. texanum*, and this infection was the most abundant occurrence of a *Synchytrium* species that I have observed so far. To cause such a wide-spread and dense infection, apparently, millions of planospores must have been present when the host plants emerged. Later, in April of 1958, I found this species in limited quantities on the same host along a roadside near Bastrop, Texas, 30 miles south of Austin. I visited the same locality at Austin again on May 6, 1959, but found only a sparse infection of *P. rhodosperma*. Obviously, the abundance of the fungus varies from year to year and is correlated with the degree of resting-spore germination, rainfall in the spring, and the number of susceptible host plants present.

At first I believed that this species might be identical with *S. macrosporum* Karling which occurs abundantly on several hosts in central Texas, but a study of its development, host range and host reaction has convinced me that it is a different species for which I propose the name *S. texanum*.

Synchytrium texanum sp. nov.

Sporis perdurantibus sphaericis, 110–250 μ diam. vel subsphaericis, ovalibus, 102–170 $\times$ 120–215 μ diam.; parietibus laevibus, brunneis, 5–7 μ crassis; soris sphaericis, 180–370 μ diam. vel subsphaericis, 120–260 $\times$ 180–285 μ diam.; sporangiis numerosis, 40–500, polyhedris, 22–27 μ diam.; zoosporis ovalibus, 3 $\times$ 4 μ diam., oblongis, 2–2.5 $\times$ 5–6 μ.

Resting spores usually solitary, sometimes 2 in a cell, spherical, 110–250 μ, subspherical, ovoid, 102–170 $\times$ 120–215 μ, with smooth, amber-brown exospore, 4–5 μ thick, and a hyaline endospore, 2–2.5 μ thick;

[1] This study has been supported by a grant from the National Science Foundation.

enveloped by a thick, dense, and hard layer of residue which fills remainder of host cell; functioning as prosori in germination. Sori spherical, 180–370 μ, or subspherical, 120–260 × 180–285 μ, with hyaline cellulose walls. Sporangia up to 500 per sorus, polyhedral, 22–27 μ in greatest diameter, exceptionally large ones up to 40 μ, with hyaline walls and orange-red content. Planospores ovoid, 3 × 4 μ, or oblong, 2–2.5 × 5–6 μ, with tapered anterior ends when actively swimming, and a light-orange refractive globule. Flagellum 12–14 μ long with a whip-lash tip. Planospores frequently functioning as gametes and fusing.

Compositely monogallic, resting-spore galls abundant on both surfaces of leaves and petioles, frequently confluent, hard, dark-brown to black when mature, protruding, usually with a flattened apex, 280–416 μ high by 320–702 μ broad; sheath 4–7 cells thick, walls of apical epidermal and inner sheath cells frequently thickened and lignified.

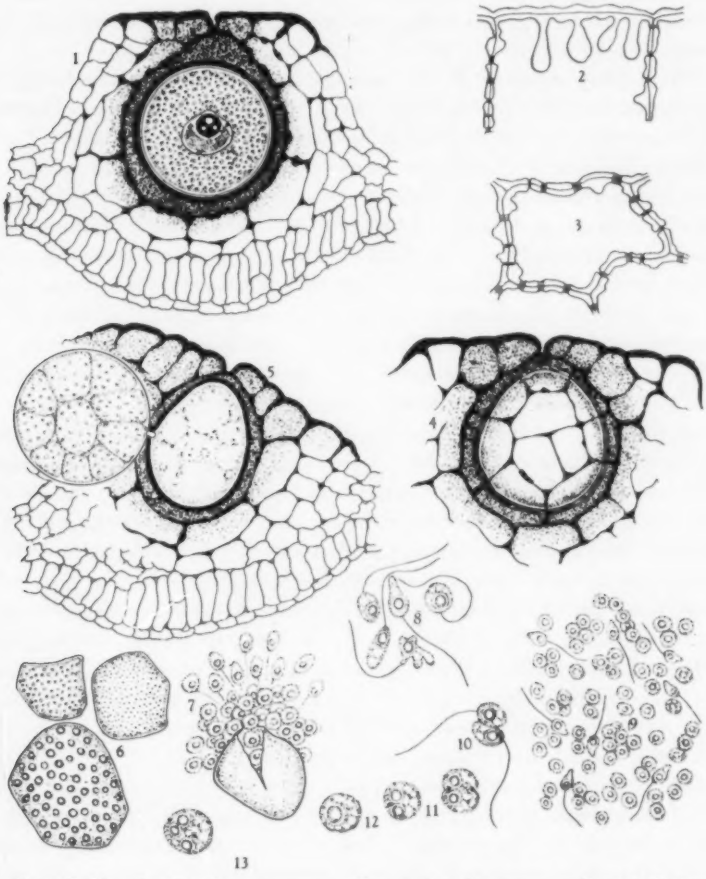
Type specimen: April 21, 1958, Arthur Herbarium, Purdue University.

On *Plantago rhodosperma*, Austin and Bastrop, Texas.

In addition to causing development of large composite galls (FIG. 1), *S. texanum* induces other marked cellular changes in the host cells surrounding the infected one. These include a significant thickening and lignification of the walls of the epidermal cells in an almost circular area or plaque at the apex of the galls. The outer wall of such cells is greatly thickened and lignified, and from it may develop tyloses or plugs which project into the lumen of the cell (FIG. 2). In addition the lateral walls are thickened also and may have numerous pitted ducts (FIG. 3). The inner sheath cells adjacent to the infected one also become enlarged with irregularly thickened and lignified walls. Furthermore, these cells as well as the epidermal and infected cells are filled with a brownish-black substance or residue which is very hard in texture and seems to function as a cement in holding the cells together. As a result it is very difficult or almost impossible to dissect out or free the resting spores from the galls. After the galls have been soaked in water for several weeks, the plaque of epidermal cells with thickened walls may be lifted up, and with it come out the resting spore and infected cell surrounded by a broken layer of inner sheath cells with thickened walls as shown in FIG. 4. This removal leaves a hole in the gall, so that it appears distinctly cup-shaped. Accordingly, the adherence of the epidermal plaque and inner sheath cells to the infected cell and the spore when the galls are dissected is characteristic for *P. rhodosperma* when parasitized by *S. texanum*.

Usually, the mature galls protrude outward at the base also so that the height of the gall may be 3 to 4 times the thickness of the leaf (FIG.

1). The basal protrusion results primarily from elongation and occasional division of the palisade cells (FIG. 1), or the multiplication and



FIGS. 1–13. *Synchytrium texanum*. FIG. 1. Resting-spore gall with flattened apex showing thickened and lignified epidermal and inner sheath cell walls. × 450. FIGS. 2, 3. Epidermal cells with irregularly thickened and lignified walls, tyloses, and pitted ducts. × 250. FIG. 4. Resting spore with surrounding layer of thick-walled inner sheath cells and apical plaque of epidermal cells. × 450. FIG. 5. Germinated resting spore, the sorus of which has ruptured and burst out of side of gall. × 450. FIG. 6. Variations in sizes of sporangia. × 120. FIG. 7. Discharge of planospores. × 120. FIG. 8. Planospores. × 360. FIG. 9. Clustering of gametes. × 120. FIGS. 10, 11. Stages in fusion of gametes. × 360. FIG. 12. Zygote. × 360. FIG. 13. Zygote from fusion of three gametes. × 360.

enlargement of mesophyll cells, depending on which surface of the leaf infection occurs. It is apparent, therefore, that the fungus stimulates not only enlargement of the infected cell, lignification and thickening of cell walls, but elongation, enlargement and multiplication of adjacent healthy cells as well.

The resting spore of *S. texanum* functions as a prosorus when it germinates, but germination takes a longer time than in *S. macrosporum*. Spores which were thoroughly soaked in water and then frozen for a week on May 21, 1958, did not begin to germinate until November 17, 1958—more than 6 months later. Another culture was prepared and treated in the same manner on Oct. 30, 1958, and some of these spores germinated 70 days later. A third culture of spores treated in the same fashion germinated in 71 days. On the other hand, mature spores of *S. macrosporum* may begin to germinate in 4 to 7 days after they have been soaked in water, and I believe that the longer time required by spores of *S. texanum* for germination is one of several differences which distinguish it from the former species.

Apparently, because of the plaque of epidermal and surrounding sheath cells with thickened and lignified walls noted above, which seems resistant to rupture, the developing sorus is unable to push out of the apex of the gall as germination progresses. Instead, it usually ruptures the side of the infected cell and pushes through the sheath cells at the side of the gall. In this process the plaque of epidermal cells may be lifted up slightly as shown in FIG. 5. In rare instances the sorus may burst out of the base of the gall, or lie obscured among the ruptured palisade and mesophyll cells. In most instances, at least, it is difficult to determine from observations of the surface of the gall whether or not the spores have germinated, and it is necessary usually to lift up the epidermal plaque to determine this with certainty.

The content of the incipient sorus cleaves into segments which are transformed into sporangia (FIG. 5). In exceptionally large sori up to 500 sporangia may be formed, and these may vary considerably in size (FIG. 6). When mature sporangia are mounted in fresh charcoal-treated water, they undergo sporogenesis in 1 to 2½ hours and produce a large number of planospores (FIG. 7). These swim about actively as oblong bodies with a slightly tapered anterior end and eventually come to rest (FIG. 8).

Careful observations have been made to determine whether or not such planospores function as gametes and fuse. It was found that within 20 to 40 minutes after emerging from the sporangia the planospores may cluster in one or several areas in the mount. Such areas may include

one to several hundred quiescent planospores which occur singly, in pairs, threes, fours, or larger groups. A portion of such an area is shown in FIG. 9. Actively swimming planospores may dart through such areas and disturb the quiescent ones, or some of the latter may twirl about and resume active motility themselves. Within 2 to 4 hours, fusions occur between pairs (FIGS. 10–12) and sometimes between 3 gametes (FIG. 13) in such areas to form biguttulate or triguttulate (FIGS. 11, 13) zygotes. Later, the refringent globules coalesce to form a larger one (FIG. 12). Following a large number of fusions in the areas noted above, the unfused planospores and gametes may resume motility, and in some cases the areas are no longer recognizable as such except for the presence of numerous zygotes. Whether or not the resting spores develop only from zygotes has not been determined as yet.

In an effort to distinguish *S. texanum* more sharply from *S. macrosporum* and other species reported on *Plantago*, host range studies were begun as soon as the resting spores germinated in the laboratory. Seeds of various plants were sown in pots in the greenhouse, and as the seedlings emerged they were inoculated. In preparing the inoculum sori of sporangia were dissected out with a micromanipulator and mounted in water on slides, and within ¾ to 2 hours they produced thousands of swarming planospores. These were transferred by pipette directly to wetted leaves of seedlings, and the latter were covered by bell jars to maintain a high humidity for 8 to 20 hours. Each of the seedlings were inoculated several times over a period of several weeks. The results of these tests are shown on page 26. Plus and minus signs after each species indicate success or failure of infection, the bold numbers indicate the number of seedlings inoculated, and those in parenthesis indicate the number of times each seedling was inoculated.

So far 51 species in 47 genera of 33 families have been inoculated, and of these only *Plantago virginica, Heliotropium indicum, Pisonia aculeata* and *Xanthium strumarium* became infected, although some seedlings were inoculated 4 to 15 times with suspensions of planospores. In all of the infected species most of the galls and spores aborted before attaining maturity. The results of these inoculations indicate that *Synchytrium texanum* may have a limited host range, and this is substantiated by its absence on other plants growing among the heavily infected *Plantago rhodosperma* in Texas in 1958. The plants included *Sonchus* sp., *Osmorhiza claytoni, Trifolium repens, Lespedeza* sp., *Galinsoga parviflora, Smilax* sp., *Cirsium palustre, Taraxacum officinale, Anemone* sp., *Dichondra repens, Tragia urticifolia, Houstonia* sp., *Oxalis* sp., *Specularia trifoliata* and *Urtica chamaedryoides*. None of

Moraceae
Chlorophora tinctoria −, **6**, (8)
Urticaceae
Urtica chamaedryoides −, **6**, (8)
Caryophyllaceae
Cerastium vulgatum −, **5**, (8)
Stellaria media −, **8**, (6)
Basellaceae
Basella alba −, **2**, (6)
Amaranthaceae
Amaranthus berlandieri −, **3**, (8)
Chenopodiaceae
Kochia childsii −, **20**, (8)
Nyctaginaceae
Boerhaavia paniculata −, **4**, (6)
Pisonia aculeata +, **2**, (6)
11 galls on one leaf, all but one of which aborted
Phytolaccaceae
Phytolacca americana −, **4**, (8)
Cruciferae
Hesperis matronalis −, **12**, (8)
Rosaceae
Duchesnia indica −, **6**, (8)
Leguminosae
Acacia occidentalis −, **2**, (6)
A. tortuosa −, **3**, (6)
Simaroubaceae
Ailanthus altissima −, **4**, (6)
Euphorbiaceae
Tragia urticifolia −, **2**, (12)
Anacardiaceae
Spondias mombin −, **1**, (8)
Malvaceae
Abutilon theophrasti −, **3**, (6)
Malva rotundifolia −, **5**, (8)
Modiola caroliniana −, **3**, (8)
Thespesia populnea −, **3**, (8)
Bixaceae
Bixa orellana −, **5**, (8)
Onagraceae
Gaura coccinea −, **12**, (6)
Oenothera biennis −, **5**, (8)
Umbelliferae
Daucus carota −, **2**, (6)
Hydrocotyle bonariensis −, **4**, (8)
Osmorhiza claytoni −, **3**, (5)
Asclepiadaceae
Ceropegia palustris −, **6**, (8)
Oxypetalum caeruleum −, **10**, (8)
Polemoniaceae
Cobaea scandens −, **2**, (8)
Gilia capitata −, **10**, (8)
Boraginaceae
Heliotropium indicum +, **4**, (5)
sparse infection, 8 galls on cotyledons
Tournefortia hirsutissima −, **5**, (6)
Labiatae
Physostegia virginiana +, **10**, (8)
Verbenaceae
Duranta plumieri −, **4**, (6)
Plantaginaceae
Plantago aristata −, **12**, (15)
P. lanceolata −, **1**, (5)
P. major −, **4**, (6)
P. virginica +, **1**, (15)
one aborted and one mature gall with a spore
Santalaceae
Santalum album −, **4**, (8)
Rubiaceae
Guettarda elliptica −, **2**, (6)
Caprifoliaceae
Lonicera xylosteum −, **4**, (6)
Solanaceae
Capsicum baccatum −, **4**, (6)
Scrophulariaceae
Collinsonia canadensis −, **3**, (8)
Bignoniaceae
Jacaranda filicifolia −, **1**, (6)
Cucurbitaceae
Cucumis anguria −, **2**, (5)
Campanulaceae
Specularia perfoliata −, **5**, (6)
Compositae
Anthemis tinctoria −, **10**, (8)
Centaurea imperialis −, **10**, (8)
Cirsium palustre −, **3**, (6)
Xanthium strumarium +, **2**, (6)
Sparse infection, 32 galls, 18 of which aborted

these species were infected when examined again in May, 1959 and August, 1960. In contrast, it may be noted that *Synchytrium macrosporum* is ubiquitous in host range. So far I have transferred it successfully to over 780 species in 555 genera of 150 families. Many of these plants are the same hosts which *S. texanum* failed to infect.

Several other species of *Synchytrium* have been reported as parasites of *Plantago;* but because all but one, *S. aureum* Schroeter, are incompletely known, it is not certain that they are valid species. With the exception of *S. punctum* Sorokin all are compositely monogallic, short-cycled like *S. texanum* and belong in the subgenus *Pycnochytrium* so far as their life cycles are known. These include *Synchytrium aureum* on *Plantago alpina, P. erecta, P. lanceolata, P. major, P. psyllium* and *P. virginica* (Passerini, **1881**; Schroeter, **1885**; Rytz, **1907**, **1932**; Neger, **1908**; Hammarlund, **1933**); *Synchytrium plantagineum* Sacc. & Speg. on *P. lanceolata* (Saccardo, **1878**; Overholts, **1933**; Maneval, **1937**); *Synchytrium punctum* on *P. media* (Sorokin, **1877**); *Synchytrium plantaginicola* Spegazzini (a nomen nudum) on *Plantago* (*P. media*?) sp. (Spegazzini, **1925**) and *Synchytrium* sp. on *P. tweedyi* (Raper and Cooke, **1954**; Karling, **1956**). *Synchytrium plantagineum* (type specimens) is considerably smaller than *S. texanum* and may prove to be identical with *S. aureum. Synchytrium punctum* is a minute simply monogallic species whose resting spores are reported to be only 7–20 μ in diameter, and it is quite unlike *S. texanum.* The resting spores of *Synchytrium* sp. on *P. tweedyi* also are smaller than those of *S. texanum* and induce smaller galls with no marked thickening and lignification of the epidermal and sheath cell walls. The only other species which resemble *S. texanum* are *S. plantaginicola,* which Spegazzini did not diagnose, and the *Synchytrium* collected and misidentified as *S. plantagineum* by Tracy in 1888 and Tracy and Earle in 1895 on *P. lanceolata* at Starkeville, Miss., which I described in 1956. Since discovering *S. texanum* I have re-examined carefully the herbarium specimens and stained sections of these species from Mississippi for the purpose of comparing them more closely with the Texas fungus. The resting spores of these species do not attain the maximum diameters of those of *S. texanum,* but in the medium ranges their sizes are fairly similar. Both species induce composite galls which are usually smaller than those stimulated by *S. texanum,* but this difference in size may be due in part to shrinkage from drying. Only dried herbarium specimens of *S. plantaginicola,* Tracy's, and Tracy and Earle's fungi were available for sectioning and measurement, while the measurements of *S. texanum* were made from sections of living material. All three of these fungi cause

similar thickening and lignification of the walls of the epidermal cells around the apex of the galls. Also, the walls of the enlarged inner sheath cells are irregularly thickened. As a consequence it is difficult to free the resting spores, and in dissection they usually come out with the infected cell surrounded by a layer of broken sheath cells and an apical plaque of thick-walled epidermal cells. Whether or not the host reaction is specific and can be used effectively as a diagnostic criterion is uncertain, because it occurs also to some extent in *P. lanceolata* infected by *S. plantagineum* (type material). Nonetheless, I believe that *S. plantaginicola* and the Mississippi species noted above may prove to be identical to *S. texanum*, but this must be verified by more intensive studies of their life cycles and host ranges from living material.

SUMMARY

Synchytrium texanum is a short-cycled parasite of *Plantago rhodosperma* in Texas which develops large thick-walled resting spores in composite galls. These spores function as prosori in germination and develop superficially attached sori which contain 40 to 500 sporangia. Accordingly, *S. texanum* is a member of the subgenus *Pycnochytrium*.

Seedlings of 51 species in 47 genera of 33 families were inoculated with suspensions of planospores 4 to 15 times under greenhouse conditions to test the host range of *S. texanum*, and of these only *Plantago virginica*, *Heliotropium indicum*, *Pisonia aculeata* and *Xanthium strumarium* became infected. However, most of the galls and spores on these hosts aborted before attaining maturity.

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REFERENCES

Hammarlund, C. 1933. Beiträge zur Kenntniss der Mikromyceten Flora der Provinz Skåne (Schonen). Ark. f. Bot. **25**: 1–126.

Karling, J. S. 1956. Undescribed species of *Synchytrium*. Mycologia **48**: 83–98.

Maneval, W. E. 1937. A list of Missouri fungi with special reference to plant pathogens and wood-destroying species. Univ. Missouri Stud. **12**: 1–150.

Neger, F. W. 1908. Einige mykologische Beobachtungen aus Südamerika und Spanien. Centralbl. Bakt. u. Parasit'k. II, **20**: 92–95.

Overholts, L. O. 1933. Mycological notes for 1930–32. Mycologia **25**: 418–430.

Passerini, G. 1881. Funghi parmensi enumerati. Giorn. Bot. Ital. **13**: 267–283.

Raper, K. L. and W. B. Cooke. 1954. The 1950 Foray of the Mycological Society of America. Mycologia **46**: 670–679.

Rytz, W. 1907. Beiträge zur Kenntniss der Gattung *Synchytrium*. Centralbl. Bakt. u. Parasit'k. II, **18**: 635–655.

——. 1932. Beiträge zur Kenntniss der Gattung *Synchytrium*. III. Infektionsversuche mit einem *Synchytrium* vom Typus *S. aureum* Schroet. Ber. deut. Bot. Gesell. **50**: 463–471.

Saccardo, P. A. 1878. Fungi veneti novi v. critica, ser. VIII. Michelia **1**: 243.

Schroeter, J. 1885. Die Pilze Schlesiens. Cohn's Krypt'fl. von Schlesiens **3**: 175–197.

Sorokin, N. 1877. Ueber *Synchytrium punctum*. Hedwigia **16**: 113.

Spegazzini, C. 1925. Ficomycetea Argeninos. Rev. Argent. Bot. **1**: 87–93.

CHARACTERISTICS OF THE GENERATIVE HYPHAE OF POLYPORES OF NORTH AMERICA, WITH SPECIAL REFERENCE TO THE PRESENCE OR ABSENCE OF CLAMP-CONNECTIONS

ALCIDES RIBEIRO TEIXEIRA

(WITH 14 FIGURES)

The term *generative hyphae* was first used by Corner (1932), when he described the fruit body of *Polystictus xanthopus*, to designate the secondary (dicaryotic) hyphae which produce all other structures of the carpophore. These other structures include the specialized branches, normally much wider than the generative hyphae, thick-walled, of limited growth, commonly unbranched and aseptate, growing longitudinally in the flesh, never showing clamp-connections, to which he gave the name of *skeletal hyphae;* the still shorter specialized branches, thick-walled to solid, normally freely branched, with branches tapering and interwoven, also never showing clamp-connections, to which he gave the name of *binding hyphae;* and basidia, cystidia, setae, and any other special structures commonly found in the carpophores of polypores. Since then this term has been used by many mycologists throughout the world, becoming quite well established.

The generative hyphae are essential to the biological cycle of the polypores, necessary for the formation of the carpophores, and always present in all fruit bodies. While still undifferentiated, they are always hyaline, very thin-walled and more or less alike in all fruit bodies, except for presence or absence of clamp-connections. In some species their cells reproduce themselves with the formation of clamp-connections, in which case the cells are always dicaryotic. In other species the cells reproduce themselves with the formation of simple septa, in which case the cells may be either dicaryotic (in some species), or plurinucleate (in others), or even may be dicaryotic and plurinucleate in a single specimen. In both instances, the generative hyphae may have no capacity to form skeletal and (or) binding hyphae, or any kind of special structures (setae, cystidia, etc.), or even of forming wall pigmentation—all these being genetically constant characters for each species. Or the genera-

tive hyphae may have that capacity in different degrees, so that in some species they can only become inflated, or develop colored walls, while in other species they can produce either skeletal or binding hyphae (*dimitic* species), and in others both skeletal and binding hyphae (*trimitic* species), as well as some other special structures—all characters also being genetically constant for each species.

These hyphae can be easily studied in monomitic specimens because, of course, all hyphae present in their fruit bodies are generative. But the situation is quite different when one is dealing with dimitic or trimitic specimens. Here the generative hyphae normally collapse and disappear in the mature context and dissepiments, leaving behind the skeletal and (or) the binding hyphae, which often are colored and much thicker than the generative. The latter are the only ones easily seen under the microscope.

The great majority of descriptions of the hyphae of polypores refer to the skeletal and (or) binding hyphae. Most descriptions of dimitic or trimitic species completely ignore the generative hyphae. Even more modern mycologists have described species without clamp-connections, when in fact they possess such structures. The reason those species were described without clamps is only that the generative hyphae (the only ones capable of showing clamp-connections) were not seen.

The study of presence or absence of clamp-connections in the generative hyphae of polypores is of particular interest because of the importance of these structures in the phylogeny of the Basidiomycetes. If we accept the theory that the clamp-connections of the Basidiomycetes are homologous to the hooks or croziers of the ascogenous hyphae of the Ascomycetes, then we must think that either one class evolved from the other or both classes evolved from a common ancestor possessing these structures. It is now generally accepted not only that the Basidiomycetes must have arisen from the Ascomycetes, and from ascomycetous forms in which the ascogenous hyphae possessed croziers, but also that the ancestral basidiomycete group must have been one in which at least some members possessed clamps (cf. Rogers, **1934**, Linder, **1940**, Bessey, **1942**, and Jackson, **1944**). Therefore, clamp-connections are one of the most important clues to the evolutionary origin of the Basidiomycetes; they are structures much more primitive than basidia, and already existed before any ascus had evolved into a basidium; hence the importance of the study of these structures in one of the most advanced groups of the Basidiomycetes—the Polyporaceae—to determine which species or groups of species still show clamp-connections, and therefore retain the ancestral way of reproducing their dicaryotic cells.

MATERIALS AND METHODS

This study has been done with the aid of specimens from the herbarium of The New York Botanical Garden. The species were chosen from those listed by Overholts (**1953**) as characterized by having hyphae "with no cross walls or clamps," or "with some cross walls but no clamps." The reason for choosing this publication as the guiding reference was that it is the most recent and most nearly complete book on the pileate polypores of North America.

The best material for study would be fresh, living carpophores, collected during the growing season. However, these were not available and herbarium specimens were used entirely.

Certain areas of the fruiting body were taken for study. These are the adult tissue of the flesh, sometimes uniform, sometimes duplex; the growing margin, showing the initiation of all hyphae and other structures; the intermediate region, showing the construction of the flesh as derived from the margin; the structure of the surface, which can be studied in relation to the generative hyphae; the dissepiments and any hymenial or extrahymenial structures which may be present, their origin being traced to generative hyphae; and the context immediately above the tubes, often more compact and in some cases with extrahymenial setae whose origin can be traced to generative hyphae.

Normally, an examination of the flesh will be enough to determine if the specimen is monomitic, dimitic or trimitic. If it is monomitic, then there will be an abundance of generative hyphae to study and describe. But if it is dimitic or trimitic, possibly most of the generative hyphae are gone, and so one must look for them in the margin, which is not so easy when one has to work with old herbarium specimens.

For bringing out septation and clamp-connections, Martin's (**1934**) KOH-phloxine solution was used. This is excellent for observing young hyphae, as well as for observing characteristics of basidia, cystidia, spores, etc. It does not stain the cell wall, unless in high concentration, and then details may be obliterated.

From each area a little portion was taken with care to extract it following the longitudinal direction of the hyphae. This portion was put on a slide with one or two drops of distilled water (or the mounting solution). With the slide under a binocular at about 50 ×, and with the help of two sharp needles, the hyphae were carefully teased apart, so that they became quite separated from each other, making easier the study of their dimensions and morphology (cf. Teixeira, **1956**).

As most of the species which have been described as having hyphae

with no cross walls or clamps are either dimitic or trimitic, it was necessary to look for generative hyphae mainly in the very margin of the pileus.

RESULTS

The following descriptions deal only with the generative hyphae, with occasional reference to the skeletal or binding ones. These last two kinds of hyphae are much more difficult to describe than the generative. They will be the subject of another paper. These are partial results, as this is first of a series of papers to be published on the subject.

CORIOLUS VERSICOLOR (L. ex Fr.) Quélet — FIG. 1

Generative hyphae are better seen in the growing margin, also in the denser zone near the trichodermous cover of the surface of the pileus. They are hyaline, very thin-walled, *nodose-septate,* with simple clamp-connections (FIG. 1 *a*), most collapsing in the context, a few becoming solid (FIG. 1 *b*) and producing slender and flexuose binding hyphae, still hyaline (FIG. 1 *c*).

DAEDALEA QUERCINA (L. ex Fr.) Fries — FIG. 2

Generative hyphae are easily found in the growing margin of the pileus. They collapse when mounted in solution of Methyl-blue, but become well differentiated when mounted in KOH-phloxine, staining deeply. They are always hyaline, very thin-walled, averaging 2–2.5 μ diam., branched, *nodose-septate,* with simple clamp-connections.

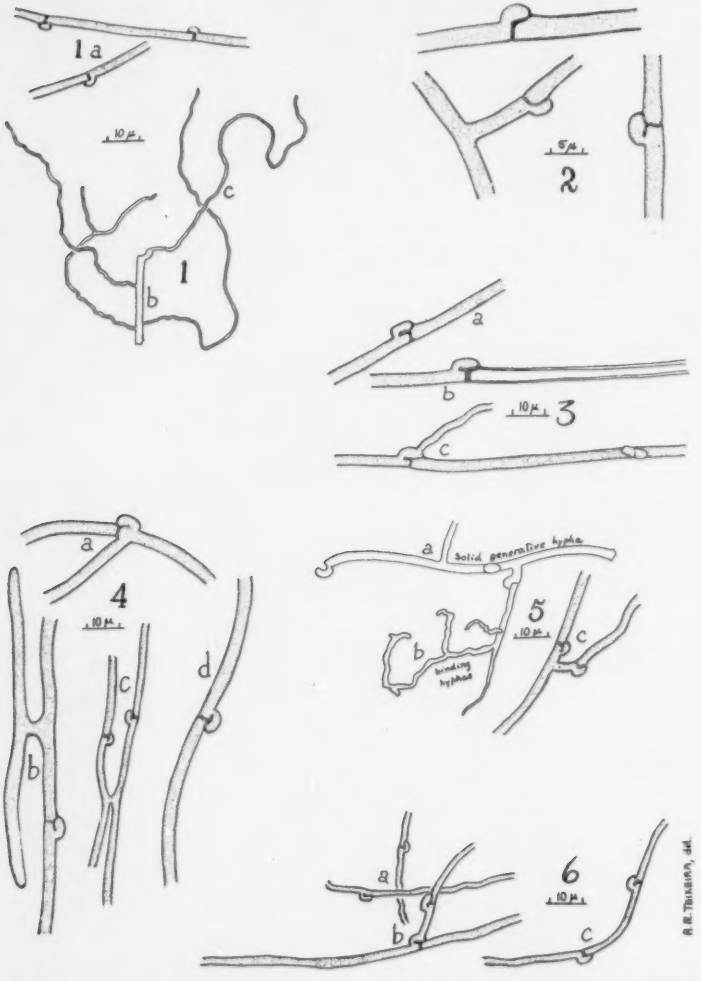
ELFVINGIA APPLANATA (Pers. ex Wallr.) Karsten — FIG. 3

Generative hyphae are difficult to find and are better seen in the growing margin of the pileus. They soon collapse and disappear, after producing the specialized branches (arboriform skeletal hyphae) which constitute most of the context and dissepiments. They are hyaline, thin-walled, very little branched, about 2.5–4 μ diam., *nodose-septate,* with simple and conspicuous clamp-connections (FIG. 3 *a, b, c*), some branching through the clamps (FIG. 3 *c*). FIG. 3 *b* shows a generative hypha giving rise to a specialized branch (skeletal hypha).

LARICIFOMES OFFICINALIS (Vill. ex Fr.) Kotl. & Pouzar — FIG. 4

Generative hyphae are easily found in the growing margin of the pileus, as they collapse and disappear early. They are hyaline, very thin-

walled, little branched, 2.5–3.5 μ diam., some as wide as 4 μ, and some as narrow as 2 μ, *nodose-septate,* with simple and conspicuous clamp-connections (FIG. 4 *a–d*). A few *H*-hyphae can also be found (FIG. 4 *b, c*).



FIGS. 1–6. Generative hyphae. FIG. 1. *Coriolus versicolor.* FIG. 2. *Daedalea quercina.* FIG. 3. *Elfvingia applanata.* FIG. 4. *Laricifomes officinalis.* FIG. 5. *Fomes fomentarius.* FIG. 6. *Fomes marmoratus.*

FOMES FOMENTARIUS (L. ex Fr.) Kickx. FIG. 5

Generative hyphae are hyaline, thin-walled, slender, flexuose, staining deeply in phloxine, narrow, averaging 2–3 μ diam., some as thin as 1.5 μ, others as wide as 3.5 μ, with conspicuous but not abundant clamp-connections, one in every 70 to 90 μ of hyphal length (FIG. 5). They are little branched, the branches normally being formed near a clamp-connection (FIG. 5 *c*). Most of them collapse and disappear in the adult context, but some become solid or sub-solid and slightly brownish (FIG. 5 *a*), after giving rise to the intricate, short-branched binding hyphae (FIG. 5 *b*).

FOMES MARMORATUS (Berk. & Curt.) Cooke FIG. 6

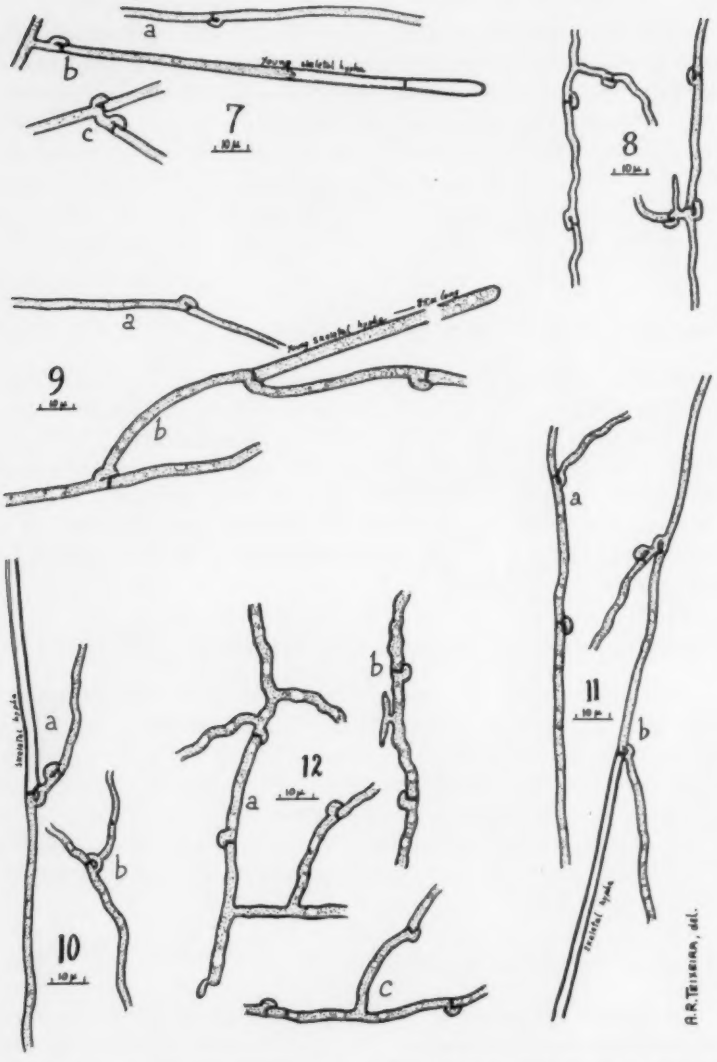
Generative hyphae are hyaline, thin-walled, slender, flexuose, staining deeply in phloxine, very narrow, averaging about 2 μ in diameter, some even narrower (1.5 μ diam), a few reaching up to 3 μ diam. They soon collapse and disappear in the context and dissepiments, and are better seen in the growing margin of the pileus. They are freely branched, *nodose-septate,* with simple clamp-connections, sometimes branching from the clamp (FIG. 6 *b*). Clamp-connections rather inconspicuous (FIG. 6 *a–c*).

FOMITOPSIS PINICOLA (Swartz ex Fr.) Karsten FIG. 7

Generative hyphae are always hyaline, contents staining deeply in phloxine. They average from 2.5 to 3.5 μ in diameter, are *nodose-septate,* with simple clamp-connections (FIG. 7 *a–c*), little branched, mostly long and flexuose, intermingled with the skeletal hyphae in the context of the growing margin of the pileus, collapsing in the older part of the context and dissepiments. FIG. 7 shows a young specialized branch (aciculiform skeletal hypha) coming from a clamp-connection of a generative hypha.

DAEDALEA CONFRAGOSA (Bolt. ex Fr.) Fries FIG. 8

Generative hyphae are always hyaline, very thin-walled, collapsing in the context and dissepiments. They are not easy to find in the carpophore, except in the growing margin of the pileus. They are rather thin, averaging about 2–3 μ diam. They are *nodose-septate,* with abundant and conspicuous clamp-connections, freely branched, sometimes showing complex branching.



FIGS. 7–12. Generative hyphae. FIG. 7. *Fomitopsis pinicola.* FIG. 8. *Daedalea confragosa.* FIG. 9. *Daedalea juniperina.* FIG. 10. *Gloeophyllum abietinellum.* FIG. 11. *Gloeophyllum striatum.* FIG. 12. *Fomitopsis ohiensis.*

DAEDALEA JUNIPERINA (Murrill) Murrill FIG. 9

Generative hyphae are easily seen in the growing margin of the pileus, either in the context or in the dissepiments. They are always hyaline, very thin-walled, freely branched, with the contents staining deeply in phloxine. They average about 2.5 to 3.5 μ in diameter, are *nodose-septate*, with simple and conspicuous clamp-connections (FIG. 9 *a*, *b*), often branching from the clamps (FIG. 9 *b*).

GLOEOPHYLLUM ABIETINELLUM Murrill (Type specimen seen) FIG. 10

Generative hyphae are easily found in the margin of the pileus. They are always hyaline, very thin-walled, with contents staining deeply in phloxine, freely branched, often branching from the clamps (FIG. 10 *a*, *b*). They average about 3–3.5 μ diam., and show *abundant and conspicuous clamp-connections*. FIG. 10 *a* shows the origin of a skeletal hypha.

GLOEOPHYLLUM STRIATUM (Swartz ex Fr.) Murrill FIG. 11

Generative hyphae are easily found either in the context or in the dissepiments near the margin of the pileus. They are always hyaline, very thin-walled, with contents staining deeply in phloxine, and averaging about 3–3.5 μ in diameter. They are *nodose-septate*, with abundant and conspicuous clamp-connections, branching from the clamps (FIG. 11 *a*, *b*). FIG. 11 *b* shows also the origin of a skeletal hypha.

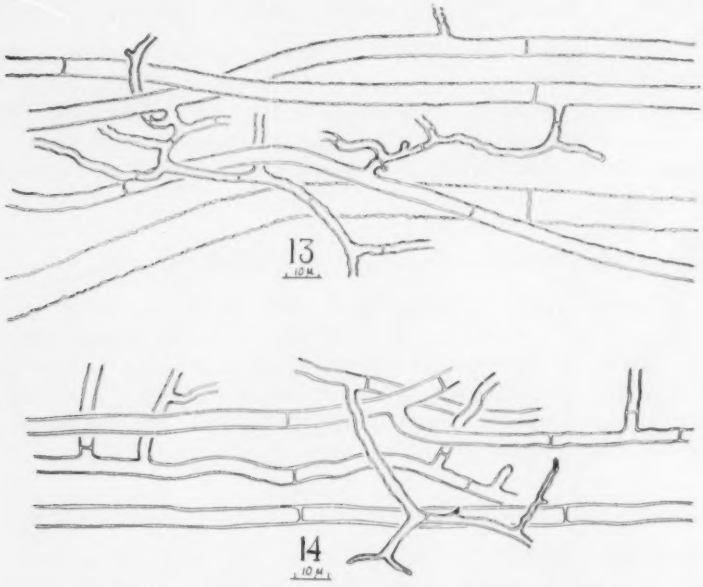
FOMITOPSIS OHIENSIS (Berk.) Singer FIG. 12

Generative hyphae are easily found in the dissepiments, and are better seen near the margin of the carpophore. They are always hyaline, very thin-walled, with contents staining deeply in phloxine, normally undulate, flexuose, averaging about 3–4 μ in diameter, *nodose-septate*, with abundant but not conspicuous clamp-connections (FIG. 12 *a*, *b*, *c*). Much branched, branching at right angle between clamps.

INONOTUS HISPIDUS (Bull. ex Fr.) Karsten FIG. 13

Monomitic species, only generative hyphae being present. These are yellowish-brown (only the growing tips are hyaline), becoming emerald green when mounted in Methylene-blue solution. *No clamp-connections* are present in any part. The main hyphae are mostly 5–7 μ in diameter, a few found as thick as 11 μ, and some as thin as 4 μ. The walls are

only slightly thickened (lumen always much wider than the thickness of the walls), and rather corrugate, either when mounted in KOH or in acid solution. The main, longitudinally running hyphae are little branched. The branches are narrower, about 3.5–5 μ diam., up to 6 μ diam., freely branched and septate, interwoven, growing in any direction. Many "bridge hyphae" seen, either between the main longitudinal hyphae or between these and the interwoven many-branched ones. In the dissepiments the hyphae are darker than in the context, very little branched,



FIGS. 13–14. Generative hyphae. FIG. 13. *Inonotus hispidus.* FIG. 14. *Inonotus cuticularis.*

frequently septate, mostly 3–4.5 μ diam., some up to 5.5 μ or even more in diameter, a few as narrow as 2.5 μ diam., intricate interwoven; many H-hyphae present. The hyphae of the dissepiments have smooth walls.

INONOTUS CUTICULARIS (Bull. ex Fries) Karsten FIG. 14

Monomitic species, only generative hyphae being present. These are yellowish-brown, becoming emerald green when mounted in Methylene-blue solution. *No clamp-connections present.* Main hyphae interwoven but growing more or less longitudinally in the context. They are only

slightly thick-walled, frequently branched and septate, only the growing tips being hyaline, soon becoming yellowish-brown. The longitudinal portions are wider, about 4–6 μ diam., some up to 7 μ diam., with branches which are directed either in the same direction or in any other direction, often running perpendicularly to the main hyphae. These branches can branch again several times, giving origin to some binding-like processes, with endings in the flesh, sometimes becoming darker than the main hyphae. The branches and branchlets are about 3–4 μ in diameter, tapering to 2.5–3 μ before ending.

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LITERATURE CITED

Bessey, E. A. 1942. Some problems in fungus phylogeny. Mycologia **34**: 355–379.

Corner, E. J. H. 1932. The fruit-body of *Polystictus xanthopus* Fr. Ann. Bot. (London) **46**: 71–111.

Jackson, H. S. 1944. Life cycles and phylogeny in the higher fungi. Trans. Roy. Soc. Can. 3rd Ser. Sec. V. **38**: 1–32.

Linder, D. H. 1940. Evolution of the Basidiomycetes and its relation to the terminology of the basidium. Mycologia **32**: 419–447.

Martin, G. W. 1934. Three new heterobasidiomycetes. Mycologia **26**: 261–265.

Overholts, L. O. 1953. The Polyporaceae of the United States, Alaska and Canada. i-xiv. 1–466, 132 pl. Univ. Mich. Press, Ann Arbor. (Prep. for publ. by J. L. Lowe.)

Rogers, D. P. 1934. The basidium. Univ. Iowa Stud. Nat. Hist. **16**: 160–182.

Teixeira, A. R. 1956. Método para estudo das hifas do carpóforo de fungos poliporáceos. Publ. Inst. Bot. São Paulo (Brazil). 22 pp., 15 fig.

OBSERVATIONS ON GYMNOASCACEAE. VII. A NEW SPECIES OF PSEUDOARACHNIOTUS FROM HONDURAS SOIL

HAROLD H. KUEHN AND ROGER D. GOOS

(WITH 18 FIGURES)

During a study of fungi found in the rhizosphere of the cultivated banana, *Musa sapientum L.*, "gros Michel," in Honduras, one of the authors (RDG) isolated a species of *Pseudoarachniotus* which proved to be new to science. The purpose of this paper is to describe this new representative of the Gymnoascaceae with regard to its morphological and cultural characteristics.

OBSERVATIONS

Pseudoarachniotus reticulatus sp. nov.[1]

Peritheciis absentibus; ascis in aggregatis nudatis dispergentibus in hyphis sterilibus. Ascis singulis in aggregatis mensis 27–66 μ diam., saepius confluentibus ad grandes aggregatos magnitudinis et figurae incertae; hyphis pilei rubri radiantibus ab ascis aggregatis. Ascis hyalinis plus minusve ovoideis, evanescentibus, (10)11–13.5(16.5) μ diam., fere 8-sporis, raro 2–4 sporis. Ascosporis globosis, reticulatus, fere involucro tectis magnitudinis et figurae incertae. Ascosporis pallido-flavis, 3.3–6 μ diam., extensione excludente. Hyphis sterilibus hyalinis, 1.1–3.3 μ diam., myceliis "racquet" presentibus; sporis imperfectis absentibus.

Ascocarps absent. Asci in exposed groups scattered among the vegetative hyphae; individual groups of asci measure 27–66 μ diam., but often are confluent to form larger masses of indefinite size and shape; red-capped hyphae radiate from the clusters of asci. Asci hyaline, more or less ovoid, evanescent, (10)11–13.5(16.5) μ diam., usually 8-spored, rarely 2- or 4-spored; ascospores globose, reticulate, usually with a peripheral extension or envelope of irregular size and shape. Ascospores pale yellow, 3.3–6.0 μ diam. excluding the peripheral extension. Vegetative hyphae hyaline, 1.1–3.3 μ diam.; racquet mycelium also present; imperfect spore phase lacking.

Colonies on YpSs agar (Emerson, **1941**) are at first colorless, restricted and pasty, a type of growth characteristic of many other Gymno-

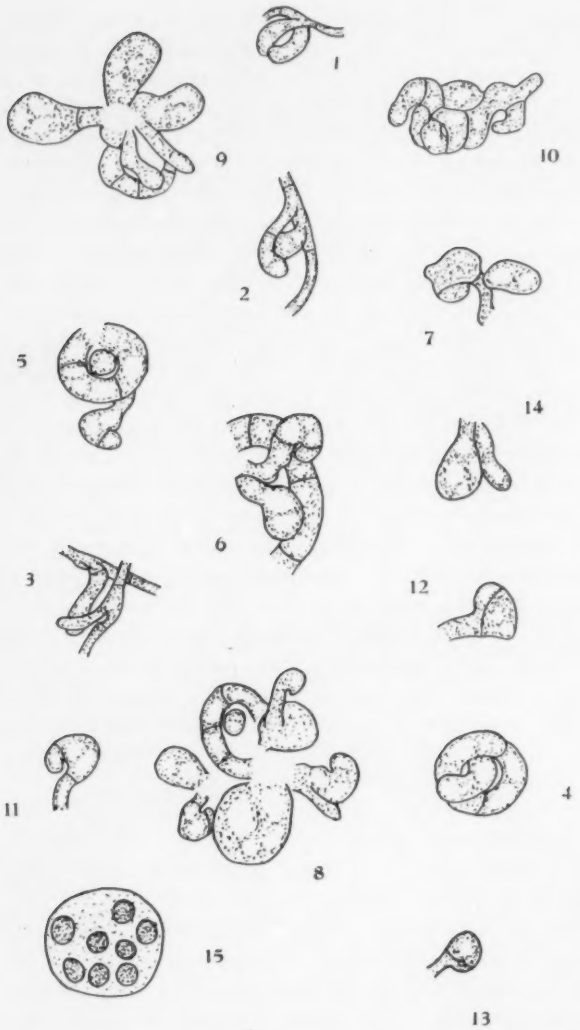
[1] We would like to thank Dr. Tibor Benedek for preparation of the Latin description.

ascaceae, such as *Gymnoascus reessii* Baran (Kuehn, **1956**). Aerial mycelium develops and becomes light orange-yellow to ochre in 10 days in older portions of the colony. This development of color is coincident with the appearance of asci. After 25 days the entire colony is tinged red-orange, although white cottony overgrowths of hyphae may be present. Aerial mycelium is limited in amount, and the colony surface is deeply furrowed. An orange-yellow exudate forms in the cottony sectors. The reverse is dull orange-yellow and furrowed. An earthy odor, typical of many members of the Gymnoascaceae, is present. Growth is such that after 26 days the surface of a Petri dish is covered if incubated at 23° C. Older colonies assume a greenish tint.

Colonies on Czapek's agar are thin, restricted and with submerged growth except for the tufts of asci. The only aerial hyphae are those in the regions of ascus development. In 15 days at 25° C the colony diameter is 6 cm, and in such a colony the extent of the region of ascus development is about 2 cm diam. The tufts of asci are pale orange in color and constitute the only color of the colony. These tufts are quite diffuse, not densely aggregated, and under a dissecting microscope can be counted with ease. The colony reverse is the color of the pale orange tufts. No odor is present. Radiating red-capped hyphae about the tufts of asci are especially prominent on this medium. In older colonies these hyphae can be seen readily under the dissecting microscope. They measure up to 77 μ long and 2 μ wide. Each is terminated by an enlargement, 2.5–5.0 μ diam., which usually is encrusted by an apical cap of red granules. All of the radiating hyphae are colored to some extent by the red apical caps. Red granules or encrustations may also be present along the walls of the radiating hyphae.

On potato-dextrose agar, at 25° C, the fungus produces white, zonate, colonies with abundant floccose and somewhat ropy aerial mycelium. Sporulation begins after 4–5 weeks, after which typical yellow-orange color appears in the sporulating areas.

Colonies on Sabouraud's maltose agar after 17 days attain 6 cm diam., with a slight pale yellow or cream coloration in older areas. Colonies are zonate although the concentric rings are partly obscured by the white aerial hyphae. The colony reverse is in shades of light brown, and is furrowed. Very few asci have developed after 17 days of incubation. After one month asci are quite prevalent in the older portions of the colony. The radiating red-capped hyphae described on Czapek's agar can also be noted. The zonate structure is completely obscured by the white aerial, cottony hyphae in older cultures. The older portions of the colony are light orange, with droplets of a pale orange to pale



FIGS. 1–15. *Pseudoarachniotus reticulatus*. FIG. 1. Two gametangial initials arising from the same parent hypha. FIG. 2. Two initials, one of which is one-septate. FIG. 3. Initials arising from different parent hyphae, with one initial delimited from the parent hypha by a septum. FIG. 4. A coil as seen from above, with the septate ascogonium around the central antheridium. FIG. 5. A coil viewed from above, showing the septate ascogonium and central antheridium at one level, with a crozier arising at a lower level from the ascogonium which is in a different

yellow exudate, and merge gradually into a white cottony margin. No odor is present. Older colonies assume a greenish tint.

Colonies on glucose-peptone medium, at 25° C, in 15 days are 3.4–4 cm diam., orange-yellow at the center, grading to white at the periphery, and showing faint zonation. Mycelium is somewhat ropy. Sporulation is moderate after 15 days of incubation.

Colonies on malt extract agar in 15 days are 5.5–6.5 cm diam., yellow-orange, with floccose white margins 1 cm wide. A golden yellow exudate is produced in moderate to abundant amounts. Aerial mycelium is ropy in colony center and sporulation is abundant. Reverse of the colony is yellow.

This fungus was isolated from rhizosphere soil of the commercial Gros Michel banana, from roots collected during July, 1958, in Omonita Farm, Tela Railroad Company, La Lima, Honduras. Cultures have been deposited with the American Type Culture Collection, Washington, D. C., and with the Centraalbureau voor Schimmelcultures, Baarn, Netherlands.

The conditions necessary for sexual reproduction in Gymnoascaceae have not been extensively studied and it was considered of interest to determine whether individual thalli are heterothallic or homothallic. Thirty-two germinated ascospores were placed on slants of glucose-peptone agar, in single spore cultures. All developed asci and ascospores, indicating that the organism is homothallic. The only earlier work which provides information in this regard is that of De Lamater (**1937**) who reported that a fungus he considered as belonging to the Gymnoascaceae produced asci and spores from single ascospore cultures.

Production of asci is initiated by the formation of gametangial initials which arise as lateral branches from the same (FIGS. 1–2) or from different (FIG. 3) parent hyphae. These initials are similar in appear-

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focal plane. FIG. 6. A septate ascogonium in one optical plane, with a young ascus and a crozier arising from the ascogonium in another plane. FIG. 7. An ascogenous hypha bearing at its apex a crozier which is beginning to elongate to form another crozier. A second crozier at right angles to the first is seen in top view. FIG. 8. This figure shows the various croziers and asci which have arisen at various levels from one coil, along with a portion of the coil. No attempt has been made to illustrate the origin of the various structures. FIG. 9. Same as Fig. 8, but with an older coil. FIG. 10. A series of croziers formed at the apex of one ascogenous hypha resulting from one original crozier growing out to form secondary and tertiary croziers. FIGS. 11–13. Stages in crozier development. FIG. 14. Two asci at different stages of development having their origin from the same crozier. The older (larger) ascus developed from the penultimate cell, while the younger ascus developed from the cell formed by fusion of the ultimate and antepenultimate cells. FIG. 15. A young ascus with immature ascospores. All figures × 1455.

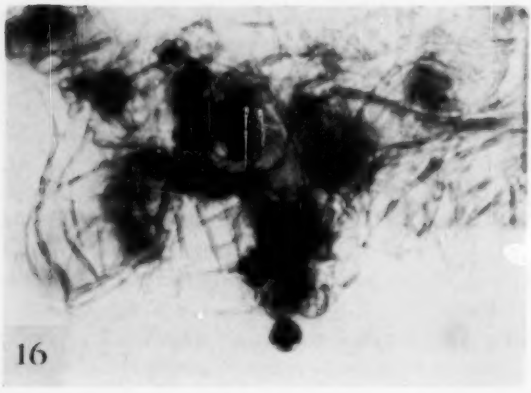

FIG. 16. Ascus cluster lying in the hyphal tangle. × approx. 500.

ance, and each becomes delineated from its parent hypha by a septum. The initials then proceed to coil about one another making one or two complete turns. The antheridium seldom becomes septate, while the ascogonium becomes divided into several cells by cross walls (FIGS. 4–6, 8).

Nothing was observed which would indicate that a fusion of the gametangia takes place. From several cells of the ascogonium ascogenous hyphae arise and soon recurve to form croziers (FIGS. 5–6, 8). The croziers either develop into asci immediately (FIGS. 6, 8–9) or the penultimate cells grow out to form additional croziers (FIGS. 7, 10).

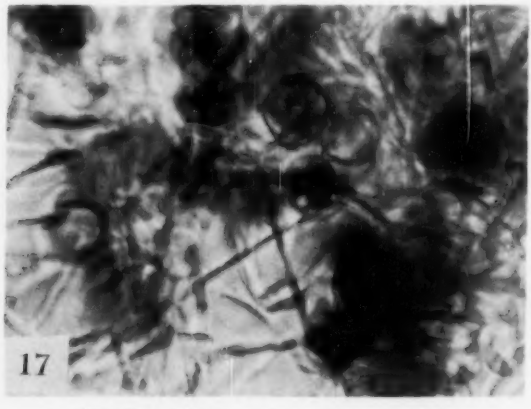

FIG. 17. Mature asci. × approx. 1,000.

In addition, the cell formed by fusion of the antepenultimate and ultimate cells often grow out to form a crozier and in this manner a group of croziers may be formed (FIGS. 7, 10). Coils were observed from which only 2 asci developed, although the average gametangial coil produced up to 10–15 asci.

No hyphae developed from the base of the coil, nor did any of the surrounding vegetative hyphae become differentiated to form a peridium. The developing asci are exposed or naked groups within the hyphal tangle.

Pseudoarachniotus reticulatus is the third species of the genus, the

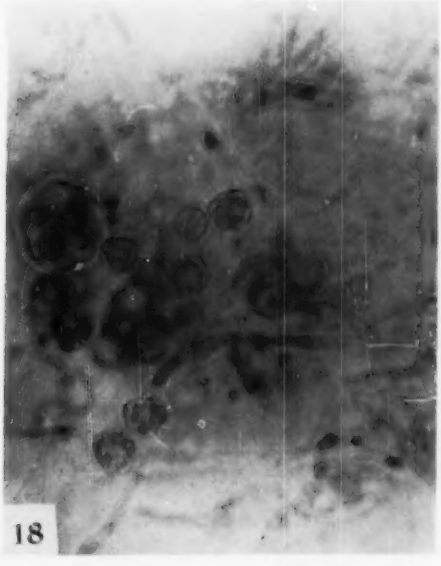

FIG. 18. Mature ascospores, showing peripheral extensions. × 1184.

others being *P. roseus* Kuehn and *P. citrinus* (Massee and Salmon) Kuehn (Kuehn, **1957**). Comparison of colony coloration among the three species permits them to be identified by color when they are grown upon a standard medium such as YpSs agar. *Pseudoarachniotus roseus* is reddish orange and *P. citrinus* is lemon yellow, while *P. reticulatus* is orange-yellow but with age becomes tinged with a red-orange color which never reaches the intensity of that produced by *P. roseus*. *P. reticulatus* can be differentiated with ease from the other two species since it has globose, reticulate ascospores, while the other species have

ovoid-lenticular or elliptical ascospores which never are reticulate. Also, *P. reticulatus* is the only species of the genus with any type of peripheral extension on the ascospore and it is, moreover, unique in possessing red-capped hyphae in the vicinity of the tufts of asci.

SUMMARY

A new species of *Pseudoarachniotus, P. reticulatus,* is described and illustrated. The developmental morphology is discussed and illustrated and the fungus is shown to be homothallic. *P. reticulatus* is distinguished from the other two species of the genus, *P. roseus* and *P. citrinus,* by its globose, reticulate ascospores, the presence of radiating red-capped hyphae among the tufts of asci, as well as by certain color differences.

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LITERATURE CITED

De Lamater, E. D. 1937. Crozier formation in the Gymnoascaceae: a preliminary note. Mycologia **29**: 187–198.

Emerson, R. 1941. An experimental study of the life cycles and taxonomy of *Allomyces.* Lloydia **4**: 77–144.

Kuehn, H. H. 1956. Observations on Gymnoascaceae. III. Developmental morphology of *Gymnoascus reessii,* a new species of *Gymnoascus,* and *Eidamella deflexa.* Mycologia **48**: 805–820.

——. 1957. Observations on Gymnoascaceae. V. Developmental morphology of two species representing a new genus of the Gymnoascaceae. Mycologia **49**: 694–706.

INCREASED SPORULATION IN FUNGI

BERNARD J. SLOAN,[1] JOHN B. ROUTIEN, AND VIRGINIA P. MILLER

(WITH 1 FIGURE)

Fungi are preserved in culture collections by several methods (1). For spore-forming fungi perhaps the freeze-dry process is the most popular and successful method since cultures can be preserved by this technique with minimal loss of viability for relatively long periods of time.

There are certain fungi, however, which cannot be freeze-dried successfully either due to lack of spore production or the presence of too few spores. These fungi must be maintained in test tubes on agar slants, kept at low temperatures, and subjected to repeated subculturing at periodic intervals. Of equal importance to the inconvenience which this procedure entails are the aspects of increased genetic instability and possibilities of contamination.

A portion of our culture collection consists of fungi which fall in the above-mentioned category. Some of these fungi have become asporogenous through repeated subculturing. Some, however, were asporogenous when obtained from our soil screening program, and some were asporogenous when obtained from reputable culture collections.

A preliminary investigation [2] of a variety of nutrients for growth-promoting effects in several fungi and streptomycetes led to the observation that certain media supplemented with coconut milk enhanced growth in some of these micro-organisms. In view of this finding and the desirability of increasing sporulation for identification, freeze-drying, and other processes requiring large numbers of spores it was decided to grow our collection of asporogenous or weakly sporogenous fungi on media supplemented with coconut milk. Fungi which still failed to sporulate were further tested on a medium containing tomato paste and oatmeal.

MATERIALS AND METHODS

Fungi. The test organisms represented a total of 424 fungi previously found to be without spores or with only a meager amount of

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[2] Work performed by senior author and Miss Irene Rollman to be published at a later date.

spores when cultivated on potato-glucose, oatmeal, or malt agars. Of this total two major taxonomic groups of fungi are represented—Ascomyceteae and Fungi Imperfecti. Diverse families belonging to these two classes are represented respectively by 170 and 254 of these organisms investigated.

Stock cultures of all fungi investigated were routinely maintained in test tubes on agar slants of potato-glucose, oatmeal, or malt, kept at 6° C after growth was established and subcultured every 6 months.

Media. In all preliminary experiments each culture was maintained in large test tubes on slants of potato-glucose agar supplemented with coconut milk. Coconut milk was used because the phytochemical laboratory at Chas. Pfizer & Co., Inc., had found it to be so useful in plant tissue-culture work, and its nutritive properties might be important for fungi.

Spot plantings to this and other media were made by transferring small pieces of agar containing mycelia from cultures to be tested. All fungi were initially incubated at 28° C for a period of from 7 to 21 days. Some fungi which were known to have special temperature requirements were later subjected to an alternating temperature of 28° C for 7 days and 20° C for 7 days. Diffuse artificial lighting occurred throughout the day.

The effect of the differently supplemented media on sporulation was determined by examination of the entire colony by use of a binocular microscope employing a magnification of 40 × or by microscopic examination of the organisms.

Preliminary experiments with the potato-glucose agar supplemented with coconut milk demonstrated that most of our asporogenous fungi grown in this manner continued to remain either sterile or to produce only a meager amount of spores and consequently could not be freeze-dried successfully. This medium, however, was found to encourage increased spore production in many sporogenous fungi of our collection.

The failure of certain fungi to produce spores on the potato-glucose agar led to testing these organisms on a medium containing alphacel and coconut milk. Many fungi were stimulated to produce spores on this medium. Those which continued to remain sterile were subsequently tested on a modified alphacel medium containing the two additions, Hunt's tomato paste (1%) and Beech-Nut baby oatmeal (1%).

Cultures forming spores on any of the media employed were freeze-dried and then reconstituted to test for viability.

Compositions of the various media employed, in grams per liter, are as follows:

(1) *Potato-glucose Agar*

potatoes *	100 g
cerelose **	10 g
agar	18 g
coconut milk ***	50 ml

pH unadjusted.
Sterilized 20 lbs/20 minutes.

* Potatoes cooked for 30 minutes in 1 liter of distilled water, strained through cheesecloth and potato water reconstituted to 1 liter with distilled water.
** A partially purified form of glucose.
*** Coconuts opened, milk pooled and filtered through several layers of cheesecloth, autoclaved 15 lbs/15 minutes and stored at 6° C until needed.

(2) *Alphacel Medium*

alphacel *	20 g
$MgSO_4$	1 g
KH_2PO_4	1.5 g
$NaNO_3$	1 g
coconut milk	50 ml
distilled H_2O	1000 ml

pH adjusted to 5.6.
Sterilized 20 lbs/20 minutes.

* Non-nutritive cellulose obtained from Nutritional Biochemicals Corp., Cleveland 28, Ohio.

(3) *Modified Alphacel Medium*

contained the above-mentioned constituents plus:

Hunt's tomato paste	10 g
Beech-Nut baby oatmeal	10 g

Fusidium assay. When the above-mentioned experiments were in progress, a species of *Fusidium* was observed to respond to certain concentrations of coconut milk by increased spore production and concomitant color change of colony from white to green. In an attempt to determine the nature of the growth and sporulation factor(s) present a culture of *Fusidium botryoideum* Corda, received from the U. S. Army Quartermaster's collection, strain No. 755, was used.[3] It was maintained on agar slants of potato-glucose containing 5% coconut milk. In assaying coconut milk the seed layer and the plates were prepared as follows: the agar slants were washed with 10 ml of sterile distilled water; large Petri dishes (150 × 20 mm) containing 30–40 ml of potato-glucose agar were evenly seeded over the surface with from 0.5 ml to 1.0 ml of the harvested suspension of spores; the seeded plates were allowed to dry at room temperature for a few minutes; the different concentrations of coconut milk, as well as various levels of a variety of other compounds tested for activity similar to that present in coconut milk, were assayed by the wet paper disc method by saturating 5–7 discs

[3] On potato-glucose agar this culture agreed well with the description of *F. botryoideum* except that the spores were larger (5.0 to 8.0 μ contrasted with the size of 4.5 μ as given by Saccardo). The spores that developed on PDA plus tomato paste were also 5.0 to 8.0 μ, but those that formed on PDA plus coconut milk were smaller, 4.5 to 6.0 μ in length. On the last two media the spores were green and, therefore, did not agree with the description of *F. botryoideum*. In spite of the difference in spore size it seemed likely to us that the organism was *F. botryoideum* rather than a new species.

(12.7 mm) with the compounds tested and placing them on the surface of the seeded agar plates; customary controls were maintained by placing discs, saturated either with sterile distilled water or with various concentrations of coconut milk when compounds other than coconut milk were being tested for activity, on all plates.

Following an incubation period of 3–5 days at 28° C all plates were examined for the presence of pink zones of increased growth or green zones of increased sporulation surrounding the discs. In a few instances measurements of the zones were made.

Preliminary compounds tested for activity similar to that present in certain concentrations of coconut milk were the following: kinetin, 1–3 diphenyl urea, yeast extract, liver extract, thymidin, and vitamin B_{12}.

Only the yeast extract and liver extract produced zones of enhanced growth (no zones of sporulation) at the particular concentrations tested. It was decided, therefore, to test a variety of vitamins, purines, and pyrimidines in the hope that one of these compounds would increase green sporulation in this *Fusidium*. A list of the solutions prepared and tested is as follows:

Solution No. 1 (pyrimidines)

ammonium uridylate
5 amino uracil
5-5 diamino uracil sulfate
4 amino pteroyl glutamic acid (amino pterin)
pyrimidine 4 carboxylic acid
4,6 dihydroxy pyrimidine
2 amino 4 methylpyrimidine
2,4 diamino 6 hydroxypyrimidine sulfate
cytosine
thymine

Solution No. 2 (pyrimidines)

6 amino uracil
5,6 diamino uracil sulfate
2 amino 4,6 pyrimidinedione
2,5 diamino 4,6 pyrimidinedione
4 amino 2,6 dihydroxypyrimidine
2,4 dihydroxy 6 aminopyrimidine
2,4,5 triamino 6 hydroxypyrimidine sulfate
4,5,6 triaminopyrimidine sulfate
pteroyl aspartic acid
isocytosine

Solution No. 3 (pyrimidines)

uracil
uracil 5 carboxylic acid
uramil
uridine
uridylic acid

Solution No. 4 (purines)

adenine
adenine sulfate $2H_2O$
adenosine
adenosine triphosphate
adenylic acid
adenylic acid (muscle)
adenylic acid adenosine 5 phosphate
cytidine
cytidylic acid
2,6 diaminopurine sulfate

Solution No. 5 (purines)

guanine (free base)
guanosine
guanylic acid (guanosine 3 phosphoric acid)
hypoxanthine (6 oxypurine)

inosine triphosphate (barium salt)
inosine acid (barium salt)
purine (Lot #410H)
uric acid
xanthine
xanthosine

Solution No. 6 (vitamins)

adermine hydrochloride (Vit. B_6)
p aminobenzoic acid
ascorbic acid (Vit. C)
biotin (Vit. H)
B_{12}
d calcium pantothenate
choline chloride
folic acid

Solution No. 7 (vitamins)

glutathione
d isoascorbic acid
niacin
niacinamide
pyridoxine hydrochloride (Vit. B_6)
riboflavin (Vit. B_2)
thiamine hydrochloride (Vit. B_1)
alpha tocopherol (Vit. E)

Whether certain concentrations of biotin could be substituted for coconut milk to induce sporulation in fungi other than *F. botryoideum* was determined by initiating the following experiment: large Petri dishes (150 × 20 mm) were prepared to contain 40 ml of either alphacel medium (minus coconut milk) or this medium containing the additives, Hunt's tomato paste (1%) and Beech-Nut baby oatmeal (1%).

Nineteen cultures which had been induced to sporulate and were successfully freeze-dried were selected for study.

Each fungus was spot planted onto two plates containing either of the two above-mentioned media. Plates were incubated at 28° C for one week. Following this paper discs saturated with 100% coconut milk or biotin (40 μg/ml) or sterile distilled water were placed at the periphery of each colony approximately 90° distant from each other. Petri plates were then returned to the 28° C incubator for one week and re-examined. Some plates were incubated for an additional week (Fungi Imperfecti at 28° C and Ascomycetes at 21° C). Examination for increased sporulation surrounding the saturated discs was made by use of a microscope.

RESULTS

Our experimental results are presented in two phases: (a) determination of the effect of coconut milk and/or tomato paste-oatmeal on stimulation to produce spores or fruiting structures (pycnidia, ascocarps) in several fungi and (b) determination of the nature of the coconut milk factor responsible for increased sporulation in *F. botryoideum* QM 755.

(a) A list of the 215 cultures tested and found to produce spores is presented in TABLE I. In TABLE II are listed the 209 cultures which were tested and which *failed* to produce spores on either of the nutritive media employed. Thus it is observed (TABLE I) that over 50% of the

TABLE I

INDUCTION OF SPORULATION ON SUPPLEMENTED MEDIA

Fungus	Spores produced on alphacel medium supplemented with 5% coconut milk[a]	Spores produced on alphacel medium supplemented with 5% coconut milk, tomato paste (1%), and oatmeal (1%)	Freeze-dry results[b]
A. IMPERFECT FUNGI			
Alternaria tenuis (1464-221E)	+		+
Basidiobotrys griseus (1464-228E)	+	+	*
Botryodiplodia theobromae (1464-80H)	+	−	−
Botryodiplodia theobromae (1464-221J)	+	−	−
Botryotrichum sp. (2335-96V)	+	+	−
Botrylis speciabilis (1464-221L)	+	+	+
Botrylis tulipae (1079-207A)	+	+	+
Brachysporium oosporium (961-34E)	+	+	−
Chaetodiplodia sp. (961-40G)	+		+
Chlamydomyces palmarum (1464-258M)	+		+
Colletotrichum lindemuthianum (2781-90B)	−	+	+
Corethropsis puntonii (CBS)	−	+	*
Corynespora cassiicola (1464-227P)	+		+
Cryptostroma corticale (1464-228F)	−	+	*
Curvularia falcata (961-264L)	+	−	*
Curvularia falcata (961-264K)	−	+	*
Curvularia lunata (1079-210V)	+	−	*
Curvularia lunata (1464-7M)	+	−	*
Curvularia lunata (1079-153J)	+	−	+
Curvularia lunata (1464-255G)	+	+	*
Curvularia lunata (1464-285J)	+		+
Curvularia lunata (961-73R)	−	+	*
Curvularia pallescens (1464-220A)	+		+
Curvularia pallescens (961-264G)	+	−	*
Curvularia pallescens (1464-230C)	+		+
Curvularia sp. (961-264C)	+	−	*
Curvularia sp. (1079-154B)	+		+
Curvularia sp. (1464-215K)	+		+
Curvularia sp. (1464-247L)	−	+	*
Cylindrocarpon sp. (2781-14G)	−	+	−
Cylindrocladium scoparium (1464-236E)	+	+	−
Cytodiplospora iliae (1464-247M)	−	+	−
Cytospora sp. (961-36P)	−	+	+
Dactylaria polycephala (2335-104A)	−	+	*
Dactylaria scaphoides (2335-104B)	−	+	+
Dactylaria thaumasia (2335-104C)	−	+	*
Dematophora necatrix (1464-221T)	+	−	*
Dicoccum sp. (961-29G)	−	+	+
Diplodia zeae (1464-76L)	+	+	*
Diplosphaerodomopsis sp. (1464-222C)	+	+	−
Endogloea sp. (961-70N)	+	−	*
Epicoccum granulatum (1464-79V)	+	−	*
Epicoccum sp. (2335-100L)	−	+	*
Epicoccum sp. (961-70D)	+	+	+
Epicoccum sp. (1464-167F)	+		+
Fumago sp. (2781-50U)	−	+	+
Fusarium coeruleum (1464-232F)	+		+
Fusarium solani (1464-222N)	+		+
Fusarium sp. (1464-172A)	+		+
Fusarium sp. (2335-96U)	+		+
Fusarium sp. (2335-106X)	+	+	−
Gliobotrys sp. (961-34D)	+	−	−
Gloeosphaera globuligera (961-72C)	+	−	−
Gonatobotrys simplex (1464-223Q)	+		+
Hansfordia grisella (1464-76B)	+	−	*
Haplosporella sp. (1464-76H)	+		+
Helicosporium lumbricoides (1464-81E)	−	+	+
Helminthosporium gramineum (961-264S)	−	+	*
Helminthosporium victoriae (1079-172N)	+		+
Helminthosporium sp. (961-264P)	+	−	−
Helminthosporium sp. (1079-83C)	+		+

[a] All cultures were planted initially on Alphacel medium supplemented with 5% coconut milk. Fungi induced to produce spores on this medium are designated by a plus sign (+). Those which failed to do so are designated by a minus sign (−). The latter fungi, as well as those that were not freeze-dried successfully, were planted on Alphacel medium containing coconut milk (5%), tomato paste (1%), and baby oatmeal (1%). (second column)

[b] Code Used: + = freeze-dried successfully; − = not freeze-dried successfully; * = freeze-drying not yet attempted.

TABLE I—*Continued*

Fungus	Spores produced on alphacel medium supplemented with 5% coconut milk[a]	Spores produced on alphacel medium supplemented with 5% coconut milk, tomato paste (1%), and oatmeal (1%)	Freeze-dry results[b]
Helminthosporium sp. (1464-276G)	+		+
Heterosporium sp. (1464-223X)	+		+
Hormiactella sp. (1464-224L)	−	+	*
Humicola grisea (1464-224N)	+	+	−
Humicola grisea (2335-98H)	+	+	+
Humicola sp. (1464-99F)	+		+
Hyalodendron lignicola (1464-224K)	−	+	*
Macrophomina phaseoli (1464-232X)	+	−	−
Mauginiella scaettae (1464-232U)	−	+	+
Mycogone perniciosa (961-28Q)	−	+	*
Myrothecium inundatum (961-73C)	+		+
Myrotrichella sp. (1464-228U)	+		+
Mystrosporium adustum (1464-232W)	+	−	−
Pachybasium candidum (1464-225B)	+		+
Paecilomyces varioti (961-172Y)	−	+	+
Papularia arundinis (1464-226L)	+		+
Papulaspora sp. (961-172T)	+ (bulbils)		+
Papulaspora sp. (1464-76F)	+ (bulbils)	−	*
Penicillium aurantiacum (2781-30G)	−	+	+
Penicillium funiculosum (1464-276J)	+		+
Penicillium purpurogenum var. *rubri-sclerotium* (2781-98J)	+		+
Periconia sp. (1464-225M)	+		+
Pestalotia dichaeta (961-65M)	−	+	+
Phialophora lagerbergii (961-27N)	−	+	+
Phialophora radicicola (1464-232J)	+		+
Phoma betae (1464-76Q)	−	+	*
Phyllosticta solitaria (1464-226C)	+	−	−
Plenodomus fuscomaculans (1464-225K)	+	+	+
Podoconis sp. (2334-39C)	+	−	−
Ramulispora sp. (2781-106N)	−	+	+
Redaellia elegans (2781-108E)	−	+	−
Sepedonium sp. (1464-85Q)	+		+
Sepedonium sp. (961-28J)	+		+
Spegazzinia tessarthra (961-72J)	+		+
Steganosporium piriforme (1464-235A)	−	+	−
Stemphylium sp. (2335-106S)	−	+	+
Trichoderma album (2335-106Y)	+		+
Trichothecium polybrochum (1464-235M)	+	+	+
Verticillium cyclosporum (2335-1A)	−	+	+
Zythia pinastri (1464-235X)	−	+	+
Sphaeropsidaceae (2335-97Y)	+		+
Sphaeropsidaceae (2781-48K)	+		+
Moniliaceae (2335-103F)	−	+	−
Moniliaceae (961-153K)	+		+
Moniliaceae (1464-262F)	+		+
Moniliaceae (2335-96Y)	+	−	−
Moniliaceae (2335-107L)	+		+
Moniliaceae (2781-40Q)	+		+
Moniliaceae (1464-232R)	+	+	+
Dematiaceae (1464-99G)	+		+
Dematiaceae (1464-84Q)	+		+
Dematiaceae (2781-44D)	+		+
Dematiaceae (2335-106W)	+		+
Dematiaceae (2335-107G)	+	+	+
Dematiaceae (2335-106B)	−	+	*
Dematiaceae (1464-85L)	−	+	+
Dematiaceae (2781-108C)	+		+
B. ASCOMYCETES			
Acanthorhynchus vaccinii (1464-237B)	+	+	−
Allescheria boydii (1464-237A)	+		+
Angelina rufescens (1464-186C)	+		+
Apostemidium guernisaci (1464-186E)	+	+	−
Ascodesmis microscopica (961-47E)	−	+	*
Ascoidea rubescens (1464-237H)	−	+	+
Ascomycete (2781-32A)	−	+	*
Ascospora rubi (1464-237E)	+		+
Ascotricha chartarum (1464-186G)	+		+
Biatorella resinae (1464-186K)	−	+	*
Bombardia lunata (1464-237J)	+		+
Byssochlamys nivea (961-43J)	+		+

TABLE I—*Continued*

Fungus	Spores produced on alphacel medium supplemented with 5% coconut milk[a]	Spores produced on alphacel medium supplemented with 5% coconut milk, tomato paste (1%), and oatmeal (1%)	Freeze-dry results[b]
Caryospora putaminum (1464-238U)	+		+
Ceratostomella ips (1464-239A)	+		+
Ceratostomella ulmi (1464-238Q)	+		+
Chaetomidium fimeti (1464-238N)	+	−	*
Chaetomium globosum (961-40L)	+	−	*
Chaetomium mollipilium (961-40S)	+		+
Chaetomium spirale (961-48E)	+	−	−
Chaetomium velutinum (961-40N)	+		+
Chaetomium velutinum (1464-237N)	−	+	+
Chaetomium sp. (961-47B)	−	+	*
Chaetomium sp. (1464-238E)	+		+
Chaetomium sp. (961-41Q)	+		+
Clathrospora diplospora (1464-238T)	+		+
Clathrospora elynae (1464-104B)	+	−	*
Claviceps purpurea (2781-104D)	−	+	+
Cordyceps capitata (1464-238R)	+	−	−
Cordyceps ophioglossoides (1464-238S)	+		+
Coryne sarcoides (1464-242B)	+	+	+
Crumenula abietina (1464-261F)	−	+	*
Ctenomyces serratus (961-40D)	+		+
Daldinia concentrica (1464-239P)	−	+	*
Dermea acerina (1464-239F)	+	−	*
Didymella applanata (1464-203A)	−	+	*
Didymosphaeria opulenta (1464-242E)	+	+	+
Dimerosporium tsugae (1464-239J)	−	+	*
Diplocarpon rosae (1464-242F)	−	+	*
Dothidea noxia (1464-261H)	−	+	*
Durandiella alni (1464-239E)	+	+	+
Endomyces magnusii (1464-105M)	−	+	+
Eremascus terrestre (961-43P)	+		+
Farlowiella carmichaeliana (1464-242K)	+		+
Fimetaria humana (1464-244W)	+		+
Fimetaria inaequalis (1464-244Y)	−	+	*
Fimetaria leucoplaca (1464-242G)	+	+	−
Gelasinospora adjuncta (1464-242N)	+		+
Glomerella cingulata (1464-242V)	−	+	+
Glomerella cingulata (1464-243B)	−	+	+
Glomerella cingulata (2267-164A)	−	+	−
Glomerella cingulata (2267-164B)	−	+	−
Glomerella cingulata (2267-164C)	−	+	−
Glomerella cingulata (J885)	−	+	−
Gymnoascus setosus (961-47A)	+		+
Hypomyces ipomoeae (1464-105T)	+		+
Hypomyces rosellus (1464-243F)	+		+
Hypoxylon pruinatum (1464-105F)	+		+
Lachnea abundans (1464-243G)	+		+
Lophotrichus ampullus (1464-243M)	+		+
Massaria platani (1464-105D)	+	−	−
Melanomma subdispersum (1464-261M)	+	−	−
Melanospora parasitica (2781-90D)	+	+	+
Mitrula paludosa (1464-261N)	+	−	*
Monascus purpureus (961-202G)	+		+
Monascus sp. (961-48A)	+		+
Monilinia fructicola (1464-81P)	+		+
Monilinia laxa (1464-81J)	−	+	+
Morchella esculenta (961-44Q)	+	−	−
Mytilidion Karstenii (1464-243U)	+	−	*
Neocosmospora vasinfecta (1464-159A)	+		+
Nummularia sp. (1464-105U)	+		+
Onygena cervina (1464-243X)	+		+
Ophiobolus cariceti (1464-187A)	−	+	*
Ophiobolus oryzinus (1464-187F)	+	+	+
Ophiobolus sativus (1464-187G)	+	−	+
Ophiobolus sp. (1464-261R)	+	−	*
Ophionectria cylindrospora (1464-243Z)	−	+	+
Petriella asymmetrica (1464-244C)	+		+
Pezicula alni (1464-244A)	−	+	*
Philocopra setosa (1464-244F)	+	−	−
Phiolia termulenta (1464-244E)	+	+	+
Pleospora jegusenis (1464-261T)	+	−	*
Pyronema confluens (961-47D)	−	+	+
Roesleria hypogea (1464-261W)	−	+	*

TABLE I—*Continued*

Fungus	Spores produced on alphacel medium supplemented with 5% coconut milk[a]	Spores produced on alphacel medium supplemented with 5% coconut milk, tomato paste (1%), and oatmeal (1%)	Freeze-dry result[b]
Saccobolus violaceus (961-47F)	+	+	+
Sclerotinia fructicola (961-47G)	+	−	*
Sclerotinia fructicola (961-40F)	+	+	−
Sclerotinia gladioli (1079-135A)	+	+	−
Sclerotinia laxa (1079-135B)	−	+	+
Sclerotinia sclerotiorum (1464-106M)	+		+
Stromatinia smilacinae (1464-261Z)	+ (sclerotia)	+ (sclerotia)	*
Teichospora pseudoplea (961-44L)	+	+	+
Tubeufia helicomyces (1464-246S)	−	+	*
Valsa ambiens (961-43C)	+	−	*

total number of fungi tested were induced to sporulate on either or both of the two supplemented media employed.

Of the 148 fungi found to produce spores on the alphacel medium supplemented with coconut milk 81 of these organisms were freeze-dried successfully. Those fungi which initially failed to freeze-dry successfully or which produced too few spores to warrant freeze-drying, as well as those which had failed to produce any spores on the above-mentioned medium, were planted on the alphacel medium supplemented with coconut milk, Hunt's tomato paste and Beech-Nut baby oatmeal. Of the 96 fungi which produced spores on this latter medium (see column 2, TABLE I) 33 were successfully freeze-dried.

It was gratifying to find that 79% of the cultures induced to sporulate well could now be freeze-dried successfully.

(b) When the paper disc-assay technique was used, large green zones of increased sporulation were produced around discs saturated with 100%, 50%, 25% and 12% coconut milk. Weak pinkish-green zones were produced around discs saturated with 6% and 3% coconut milk (FIG. 1). Therefore, 17 dilutions of coconut milk (100%) were prepared ranging in decreasing half concentrations from 50% to 0.0006% and tested for activity. Reference to TABLE III illustrates that 3% coconut milk is the minimal dilution of coconut milk capable of producing weak green zones of increased sporulation. Dilutions below this level have no effect on sporulation although the two successive lower levels did result in some enhancement of vegetative growth. Decrease in zone size was not proportionate to decreasing concentrations of coconut milk.

Different dilutions of coconut milk (10%, 5% and 1%) each in combination with kinetin at 100 ppm, 10 ppm, and 1 ppm were tested using the *Fusidium*-assay technique. Kinetin alone had no stimulatory

TABLE II

LIST OF FUNGI WHICH FAILED TO PRODUCE SPORES EITHER ON COCONUT MILK-ALPHACEL MEDIUM OR ON COCONUT MILK, TOMATO PASTE-OATMEAL ALPHACEL MEDIUM

A. IMPERFECT FUNGI

Acremonium sp. 1464-221A
Actinospora megalospora 1464-236H
Alternaria passiflorae 2781-156A
Alternaria sp. 1464-77L
Amphichaeta muscicola 1464-189E
Anguillospora longissima 1464-189F
Aristastoma oeconomicum 1464-189H
Asteroma corni 1464-258G
Bartalinia sp. 1464-77N
Blennoria sp. 1464-189M
Brachysporium sp. 1464-221N
Camarosporium orni 1464-258J
Camarosporium pellucidum 1464-258K
Candelabrum spinulosum 1464-231F
Cephalosporium sp. 961-35E
Ceratophorum setosum 1464-227L
Chaetomella sp. 1464-227U
Cicinnobolus cesatii 1464-236C
Clathrosphaerina zalewskii 1464-231G
Collonaemella sp. 1464-227H
Curvularia falcata 1079-153A
Curvularia falcata 961-264K
Curvularia geniculata 961-73E
Curvularia lunata 1079-210A
Curvularia lunata 1079-172C
Curvularia lunata 1079-255D
Curvularia lunata 1464-215J
Curvularia lunata 1079-76B
Curvularia lunata 1464-215H
Curvularia lunata 1464-255P
Curvularia maculans 1079-210Q
Curvularia maculans 1079-14B
Curvularia oryzae 1464-217K
Curvularia sp. 1464-7H
Curvularia sp. 1464-219B
Cytoplea juglandis 1464-236F
Dendrophoma obscurans 1464-221W
Dendrosporium lobatum 2781-108R
Dictyosporium opacum 1464-79M
Didymella vodakii 2781-156C
Dilophospora alopecuri 2781-156D
Diplodia alni 1464-221U
Diplosporium sp. 1464-222A
Dothiorella sp. 961-202J
Epicoccum granulatum 1464-167A
Epicoccum sp. 1464-167D
Epicoccum sp. 1464-167E
Epicoccum sp. 1464-288K
Exophoma sp. 1464-76V
Fusarium sp. 1464-172C
Gelatinosporium sp. 1464-223R
Geotrichum sp. 1464-77D
Gloeodes pomigena 1464-259Y
Helicoceras oryzae 1464-224E
Helicodendron giganteum 1464-247Z
Helicoma sp. 961-33F
Helminthosporium avenae 1079-212C
Helminthosporium dictyoides 1079-172F
Helminthosporium oryzae 1079-172E
Helminthosporium sacchari 1079-172L
Helminthosporium sativum 1079-133H
Helminthosporium sativum 1079-172J
Helminthosporium siccans 1079-172M
Helminthosporium stenospilum 1079-172V
Helminthosporium sp. 1079-9P
Helminthosporium sp. 961-264P
Hendersonia abietis 2781-156F
Heterosporium sp. 1464-224C
Hormiactella sp. 961-39E
Hyphelia sp. 1464-79K
Libertella acerina 1464-232N
Macrophoma sp. 961-73J
Macrosporium sp. 1464-80B
Macrosporium sp. 1464-233A
Monochaetia unicornis 1464-262G
Monosporium venezuelense 2335-49E
Monotosporella sp. 1464-231B
Myxofusicoccum sp. 961-69R
Pestalotia adusta 961-72D
Pestalotia bicolor 1464-79A
Pestalotia guepini 1464-77P
Pestalotia virgatula 961-70K
Pestalotia virgatula 1464-225V
Pestalotia sp. 1464-244U
Periconiella velutina 2335-104E
Phaeoscopulariopsis paisii 1464-262Q
Phaeoseptoria festucae var. *muhlenbergiae* 1464-226G
Phoma terrestris 1464-225H
Phoma sp. 1464-224V
Phoma sp. 1464-226F
Phoma sp. 1464-225A
Phomales 1464-262T
Phomales 1464-262U
Placosphaeria sp. 1464-76D
Pleurophoma sp. 1464-79L
Pycnosporium sp. 961-27F
Ramularia bellunensis 1464-262V
Rhodoseptoria sp. 1464-76U
Sclerophoma entoxylina 2781-158A
Scolecobasidium constrictum 1464-79P
Selenophoma linicola 1464-233W
Septonema sp. 961-69J
Sphaeropsis malorum 961-43E
Sphaeropsis sp. 961-70H
Sphaeropsis sp. 1464-233Y
Spondylocladium atrovirens 1079-212H

TABLE II—*Continued*

A. IMPERFECT FUNGI—*Continued*

Stagonospora meliloti 1464-247W
Stemphylium solani 2781-26Z
Stigmina platani 2781-158B
Strumella coryneoides 1464-234F
Tetracladium marchalianum 1464-235N
Vermicularia sp. 961-70G
Wiesneriomyces javanicus 1464-247X
Wojnowicia graminis 2781-156K
Moniliaceae 2335-103F
Moniliaceae 2335-107E
Moniliaceae 961-153A
Dematiaceae 2258-7-8
Mycelia sterilia 2335-105T
Mycelia sterilia 2781-6U
Mycelia sterilia 2335-100J
Mycelia sterilia 2335-100P
Mycelia sterilia 1464-85F
Mycelia sterilia 1464-94S
Mycelia sterilia 1464-99J
Mycelia sterilia 1464-84J
Mycelia sterilia 1464-88H
Mycelia sterilia 1464-88J
Mycelia sterilia 1464-90K
Mycelia sterilia 1464-88K
Mycelia sterilia 1464-94F
Mycelia sterilia 1464-91K
Mycelia sterilia 1464-91L

B. ASCOMYCETES

Adelopus balsamicola 1464-186A
Anixia parietina 1464-186D
Anthostoma dryophilum 1464-237C
Ascobolus equinus 1464-237D
Ascocalyx abietis 1464-186F
Ascospora rubi 2781-156B
Belonium excelsius 1464-186J
Bulgaria inquinans 1464-186L
Calonectria decora 2781-90A
Ceriospora caudae-suis 1464-242A
Chaetomium cochlioides 961-41B
Clathrospora diplospora 1464-261C
Claviceps purpurea 1464-238V
Clithris quercina 1464-238X
Cryptodiaporthe castanea 1464-238Y
Cryptospora cinctula 1464-261G
Cryptosporella eugenia 1464-242C
Cucurbitaria laburni 1464-242D
Dermea balsamea 1464-239H
Diaporthe oncostoma 961-47K
Diatrype disciforme 1464-239G
Dibotryon morbosum 1464-239Q
Discina reticulata 1464-239K
Durandiella fraxinia 1464-239M
Durandiella rugosa 1464-239L
Eidamella spinosa 1464-239U
Endoconidiophora fimbriati 1464-239Y
Epichloe typhina 1464-239V
Eremascus fertilis 1464-239W
Eutypella parasitica 1464-106D
Galactinia sp. 1464-105P
Guignardia bidwellii 1464-79G
Heleococcum aurantiacum 1464-243C
Helotium scutula var. *vitellina* 1464-243D
Hypocrea spinulosa 1464-243E
Lambertella viburni 1464-243H
Leptosphaeria herpotrichoides 1464-243J
Leptosphaeria heterospora 1464-243K
Letendraea eurotioides 1464-236G
Lophium mutilinum 1464-243L
Melanospora sp. 1464-243N
Mollisia cinerea 1464-243Q
Morchella esculenta 961-44D
Nectria galligena 1464-243V
Niptera pulla 1464-243W
Nummularia sp. 1464-105Q
Ocellaria ocellata 1464-261Q
Ophiobolus fulgidus 1464-187B
Ophiobolus graminis 1464-187D
Ophiobolus graminis 1079-116D
Ophiobolus graminis 1079-133K
Ophiobolus heterostrophus 1464-187C
Ophiobolus herpotrichus 1079-116C
Ophiobolus miyabeanus 1464-187E
Ophiobolus setariae 1464-187H
Peziza cerea 1464-185K
Physalospora glandicola 1464-104D
Physalospora miyabeana 961-44E
Pleospora ambigua 1464-261S
Pleospora infectoria 1464-104H
Pleospora rainierensis 1464-261U
Pleospora trichostoma 1464-104A
Pleonectria pinicola 1464-244H
Pyrenophora avenae 1079-116B
Pyronema confluens 961-47C
Schizoxylon microsporum 1464-244S
Sphaerella linorum 1464-244P
Sphaerulina trifolii 1464-244Q
Thielavia sepedonium 961-38M
Thielavia terricola 1464-245H
Trochila laurocerasi 1464-264C
Ustulina vulgaris 961-44G
Venturia inaequalis 1464-245L
Xylaria fusca 961-47N
Xylaria polymorpha 961-47J

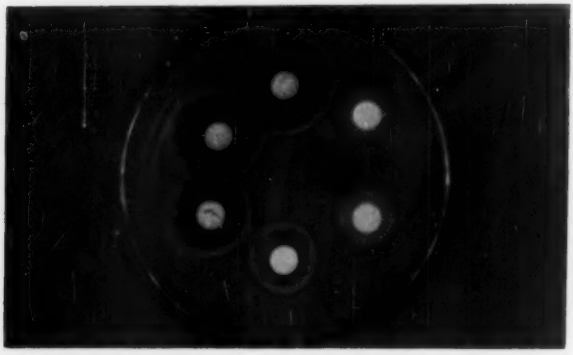

FIG. 1. Potato-glucose agar plate seeded with 0.5 ml of spore suspension of *Fusidium botryoideum* QM755. Zones of increased sporulation (green to pinkish green) are produced around paper discs saturated respectively (counterclockwise) with 100%, 50%, 25%, 12%, 6% and 3% coconut milk.

effect on growth or sporulation and also caused no enhancement of the response to coconut milk.

Negative responses (no green zones) were obtained when the following compounds were tested in the absence of coconut milk at the following concentrations: vitamin B_{12} (2 μg/ml or lower), 2,3-diphenylurea (10 μg/ml or lower), and thymidine (.01%).

Yeast extract (2% or higher) gave enhanced zones of growth but failed to produce green zones of increased sporulation. The results obtained with this compound, however, did suggest testing a variety of vitamins, purines, and pyrimidines for their ability to induce sporulation since some of these compounds are known to be present in yeast extract. A list of the compounds contained in the seven solutions prepared already has been presented. Each compound was tested at a concentration of 1 μg/ml. Green zones of increased sporulation were produced only around those discs that had been saturated with either solution number 6 or with the solutions combined in various concentrations and containing solution number 6. Since solution number 6 was a mixture of eight vitamins, each vitamin was assayed separately by the wet paper disc method. Only biotin produced green zones around the discs on the seeded plates.

The next logical step was to test a series of concentrations of biotin for activity. The following concentrations of biotin were initially tested (μg/ml): 1000, 100, 80, 60, 40, 20, 10, 2 and 1. Green zones were produced at all concentrations, and even at the lowest dilution (1 μg/ml) a green zone 36 mm in diameter was produced.

Dilutions of biotin ranging in ten-fold decreasing concentrations from 100 μg/ml to 0.00001 mμg/ml were prepared and tested for activity. Only those discs saturated with 1 μg/ml or higher of biotin produced green zones. Therefore, the minimal stimulatory level of biotin capable of inducing increased sporulation of *F. botryoideum* appears to be 1 μg/ml. It is obvious, therefore, that biotin at a concentration of 1 μg/ml will be as effective as 5% coconut milk in inducing abundant sporulation in this fungus.

An attempt was made to isolate biotin from 2½ liters of coconut milk by column chromatography. When 20% and 40% acetone was passed through a carbon-celite column containing 160 g of Darco G-60 and 80 g of celite, several eluates showed activity. The active eluates were combined and concentrated to dryness. The two isolated fractions (30 mg and 120 mg) were then subjected to infra-red analysis. Infra-red absorption spectra in potassium bromide failed to exhibit peaks similar to those exhibited by pure crystalline biotin. Apparently the two fractions contained other constituents of coconut milk which interfered with the analysis. A microbiological analysis of two samples of coconut milk (100% and 5%) revealed that the samples contained respectively 5.9 mμg/ml and 1.15 mμg/ml of biotin. Vandenbelt (3) found 20 mμg/ml of biotin present in a sample of 100% coconut milk.

TABLE III

EFFECT OF DECREASED CONCENTRATIONS OF COCONUT MILK ON PRODUCTION OF GREEN ZONES OF INCREASED SPORULATION IN *Fusidium botryoideum* QM 755. POTATO GLUCOSE AGAR PLATES INCUBATED AT 28° C FOR 3 DAYS

Per cent coconut milk	Size of zone	Color of zone
100	45 mm	Green
50	38 mm	Green
25	37 mm	Green
12.5	31 mm	Green
6	25 mm	Pink to Pale Green
3	23 mm	Pink to Pale Green
1.5	13 mm	Pale Pink
0.75	Haze, not able to measure	White
0.38	—	—
0.19	—	—
0.08	—	—
0.04	—	—
0.02	—	—
0.01	—	—
0.005	—	—
0.0025	—	—
0.0012	—	—
0.0006	—	—
Control: Sterile Distilled Water	—	—

The above-mentioned concentration of biotin present in 5% coconut milk is considerably less that the minimal concentration (1 μg/ml) required for increased spore production in *F. botryoideum*. A comparison of the latter-mentioned results with those obtained from the infra-red absorption analysis does strongly suggest that unidentifiable factors present in coconut milk (in addition to biotin) are required to produce increased sporulation.

In view of the finding that biotin apparently was *one* of the factors present in coconut milk responsible for increased sporulation of *F. botryoideum* experiments were performed to determine whether biotin was the effective substance for other fungi which we had tested.

Out of the nineteen fungi tested, eighteen failed to be stimulated by the biotin but did respond to the coconut milk. Apparently additional factors present in coconut milk are likewise required for sporulation by these fungi.

An obvious possible application of this ability of coconut milk to increase sporulation was the use of it in a medium designed for the growth of streptomycetes. Preliminary experiments showed that the addition of 5% coconut milk to Pridham's medium (dextrose 4.0 g; Difco malt extract 10.0 g; Difco yeast extract 4.0 g; distilled water to make 1 liter; adjusted to pH 7.3; agar 20.0 g) would bring about greatly increased sporulation in many strains of streptomycetes.

Experiments were performed with a small number of strains some of which sporulated not at all or very poorly. When these were planted on Pridham's plus coconut milk, the sporulation was enhanced or not altered or decreased. However, when Emerson's agar (dextrose 10.0 g; beef extract 4.0 g; peptone 4.0 g; NaCl 2.5 g; yeast extract 1.0 g; distilled water to make 1 liter; adjusted to pH 7.0; agar 15 or 20.0 g) was supplemented with 5% coconut milk, sporulation was never hampered and in over 50% of the strains it was improved.

One chytrid, *Rhizophlyctis rosea*, was grown on PDA plus coconut milk and on cherry agar plus coconut milk. The increase in number of sporangia and zoospores was extraordinary.

DISCUSSION

The original intent of this investigation was to induce sporulation in as many of our asporogenous fungi as possible. The accidental finding of a culture (*Fusidium botryoideum* QM 755) exhibiting a marked response to coconut milk by increased spore formation with concomitant color change of colony from white to green was indeed an unexpected

but interesting discovery. It enabled us to use this fungus as a neat test organism in assaying coconut milk for the presence of an unknown spore forming factor(s).

It is a well known fact that non-enriched media tend to restrict vegetative growth and in many instances to stimulate spore formation in fungi (1). It was with this concept in mind that the alphacel medium was selected, rather than other commonly used mycological media, as the medium of choice in our nutritional studies. It should be mentioned that this medium was indeed found to restrict vegetative and aerial mycelial growth and at the same time to encourage ascocarp, pycnidial and/or sporophore formation in many of the fungi tested. The alphacel medium, therefore, is definitely recommended to those interested in taxonomic, morphological, and nutritional studies. Many of those fungi which failed to produce spores or which managed to produce only a meager quantity of spores on potato-glucose and alphacel agar media produced tremendous numbers of spores when 5% coconut milk was added to the alphacel medium. Obviously coconut milk supplied some unidentifiable factor(s) which satisfied the sporulation requirements of these fungi.

Biotin alone in concentrations of 1 μg/ml or higher will cause greater sporulation by *F. botryoideum* QM 755. However, the amount of biotin in coconut milk is much less than this, so there must be some factor(s) in the milk which in conjunction with biotin will give the increased sporulation. For at least some other fungi some factor(s) other than biotin is necessary.

Several fungi which continued to remain either asporogenous or which sporulated weakly on the alphacel medium supplemented with coconut milk were successfully induced to sporulate on the alphacel medium when it contained the two additives—tomato paste and oatmeal. Apparently the tomato paste and/or oatmeal contains an unknown factor(s) which adequately satisfies the sporulation requirements of these fungi. These unknown factors either may be different from those present in coconut milk, which is probably the case, or there may be different concentrations of similar unknown factors present in coconut milk, tomato paste and oatmeal. Experimental studies conducted at our laboratory, which are not included in this report, have convinced us that it is the tomato paste and not the oatmeal which is primarily responsible for induction of sporulation in the fungi tested in this investigation. Recently (2) it has been shown that tomato extract when added to a basal medium resulted in increased growth responses in numerous basidiomycetes.

In view of the enhanced sporulation produced by coconut milk in many of the fungi studied, in several actinomycetes and a chytrid it was decided to incorporate coconut milk into the media prepared in our laboratory as a standard procedure.

Sporulation of *Fusidium botryoideum* increased when tomato paste or oatmeal was added to alphacel medium, and it seems likely that the presence of biotin in the tomato paste (traces in the oatmeal) accounts for some of this phenomenon, though microelements may also be influential.

The fungus, *F. botryoideum*, although sensitive to as low a concentration of biotin as 1 μg/ml, is far from being within the sensitive range required if it were to qualify as a test organism in standard biotin assay analyses. However, it could be used to detect the presence of higher concentrations of biotin (1 μg/ml and higher) when such concentrations are suspected of being present in certain materials. The ease with which the assay could be performed would seem to warrant its use in such a particular case.

SUMMARY

A total of 424 fungi asporogenous when grown on potato-glucose agar, oatmeal or malt were tested for spore formation on a nonenriched medium (alphacel) supplemented with either 5% coconut milk or additionally with 1% Hunt's tomato paste and 1% Beech-Nut baby oatmeal. Two hundred and forty-four were induced to sporulate on either or both of the particular media employed as follows:

(1) Fungi sporulating on alphacel medium supplemented with 5% coconut milk..... 148
(2) Fungi sporulating on alphacel medium supplemented with 5% coconut milk, 1% Hunt's tomato paste and 1% Beech-Nut baby oatmeal 96

Of the 244 fungi induced to sporulate 158 have been subjected to the freeze-dry process. One hundred and twenty-four of these 158 have been freeze-dried successfully.

Alphacel medium supplemented with 5% coconut milk is recommended as a good sporulation medium for many fungi.

Biotin was determined to be a factor present in coconut milk partly responsible for increased sporulation in *Fusidium botryoideum* QM 755.

ACKNOWLEDGMENTS

The authors wish to express appreciation to Dr. B. K. Koe and Dr. R. L. Wagner for column chromatography and infra-red absorption data.

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REFERENCES

1. **Cochrane, V. W.** 1958. Physiology of fungi. John Wiley and Sons, Inc., New York.
2. **Robbins, W. J., and Annette Hervey.** 1958. Wood, tomato and malt extracts and growth of some Basidiomycetes. Mycologia **50**: 745–752.
3. **Vandenbelt, J. M.** 1945. Nutritive value of coconut. Nature **156**: 174–175.

THE MORPHOLOGY OF AN UNDESCRIBED SPECIES OF DOTHIORA [1]

E. S. LUTTRELL

(WITH 24 FIGURES)

In the most fundamental reorganization of the Ascomycetes attempted in this century Nannfeldt (**8**) divided the Euascomycetes into two great groups, the Ascohymeniales containing forms with true perithecia or apothecia and the Ascoloculares containing forms in which the asci are borne in unwalled locules in a stromatic ascocarp. Since fungi in the Ascoloculares typically produce asci of the bitunicate type while those in the Ascohymeniales produce asci of the unitunicate type, Luttrell, who (**4**) placed primary emphasis on the more basic character of ascus structure, arrived at essentially the same division, establishing (**6**) the subclass Loculoascomycetes (Ascoloculares) for forms with bitunicate asci and usually ascostromatic ascocarps and limiting the Euascomycetes to forms with unitunicate asci and usually true perithecia or apothecia (Ascohymeniales and Plectascales). The subclass Loculoascomycetes is composed primarily of "Pyrenomycetes" in which the ascostroma is perithecium-like. It includes also a series of forms in which the ascostroma roughly resembles an apothecium, some such as those in the Hysteriaceae and the Dothioraceae having been originally classified among the Discomycetes. Müller and von Arx (**7**) assembled these forms in the new order Dothiorales, which they (**1**) referred to as "bitunicate Discomycetes." The genus *Dothiora* consequently assumes a position of critical importance in the classification of the Loculoascomycetes. Unfortunately, no developmental studies on which to base an interpretation of its structure have yet been reported.

An opportunity to determine the course of development in this group was afforded by the collection of a dothioraceous fungus on stems of *Symphoricarpos orbiculatus* Moench. at Columbia, Missouri, in 1948–49. Because the ascospores were commonly phragmosporous, this fungus was tentatively assigned to *Leptodothiora* (**6**). Material was submitted to Dr. Frans Petrak who (letter of March 21, 1956) indicated that, since the ascospores were occasionally muriform and the ascocarps were iden-

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tical with those of *Dothiora* spp., this fungus should be placed in *Dothiora*. It is in some respects intermediate between *Dothiora* and *Leptodothiora* but differs from all other species in either of these genera in its long fusiform ascospores which are deeply constricted at the middle septum and readily separate into halves. On the basis of Dr. Petrak's advice, the fungus is described as a new species of *Dothiora*, the specific name referring to the peculiar structure of the ascospores.

Dothiora schizospora sp. nov.

Ascomatibus solitariis vel gregariis, innato-erumpentibus, orbicularibus vel oblongatis, applanatis, 0.2–1.5 × 0.2–0.7 mm., contextu parenchymatico, atris, astomis, rimose vel irregulariter dehiscentibus, siccis subclausis, disco sordide albo, continuo, plano; ascis bitunicatis, aparaphysatis, subcylindraceis vel clavatis, breve pedicellatis, octosporis, 70–119 × 11–15 μ, in loculis solitariis, continuiis dispositis; ascosporis hyalinis, fusiformibus, 1–9-septatis, phragmosporis vel submuriformibus, leniter constrictis, ad medium septum profunde constrictis et facile secedentibus, 20–44 × 4–6 μ, distichis vel tristichis.

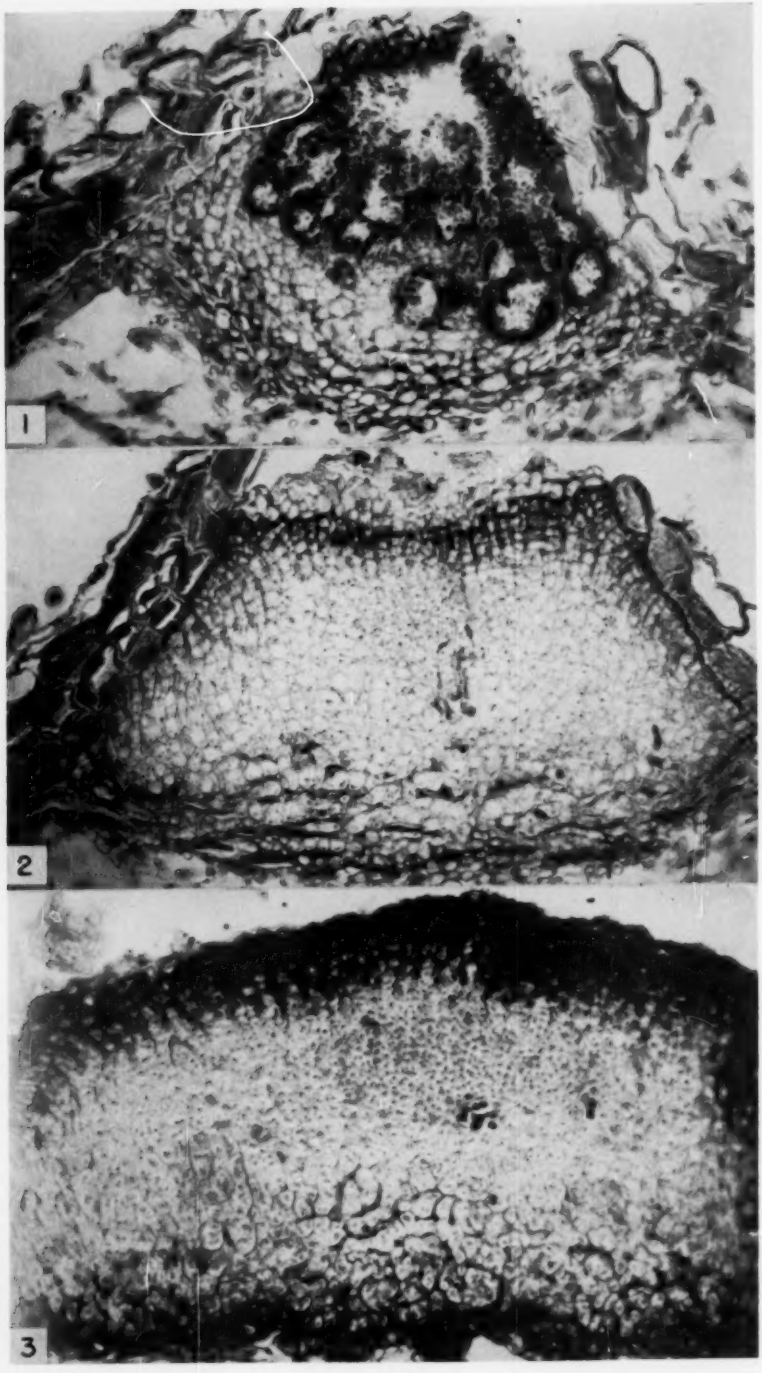
Status conidicus (*Dothichiza*): stromatibus conidiferis ascomate similis; conidiis (blastosporis) ovoideis vel oblongatis, hyalinis, continuiis, 4–9 × 2–4 μ, in loculis ex cellulis ovoideis vel pyriformibus 4.6–6 μ diam. exorientibus.

Hab. in ramis emortuis Symphoricarpi orbiculati.

The type collection (Daniel F. Millikan, Feb. 27, 1955, Columbia, Missouri) has been deposited in the National Fungus Collections, Beltsville, Maryland. Cultures isolated from the type collection have been placed in the Centraalbureau voor Schimelcultures, Baarn (catalogued as *Leptodothiora* sp. in 1957 List of Cultures, C. B. S.). My original collections in 1948 were lost, and the fragmentary collection by Dr. Millikan, on which the description is based, must serve as the type.

MATERIALS AND METHODS

In addition to the original collections, an abundant supply of *Dothiora schizospora* was obtained by placing bundles of freshly cut stems of *Symphoricarpos orbiculatus* mixed with dead stems bearing mature ascocarps in favorable locations out-of-doors in early spring, permitting natural inoculation of the fresh stems and the subsequent development of the fungus through its complete life cycle. Material was collected at intervals of two weeks throughout the year and fixed in a formalin-alcohol-proprionic acid mixture. After dehydration in tertiary butyl alcohol, the material was embedded in Fisher's Tissuemat and was sectioned at a thickness of 8 μ. Staining was with Haidenhain's hematoxylin differentiated in a saturated solution of picric acid.



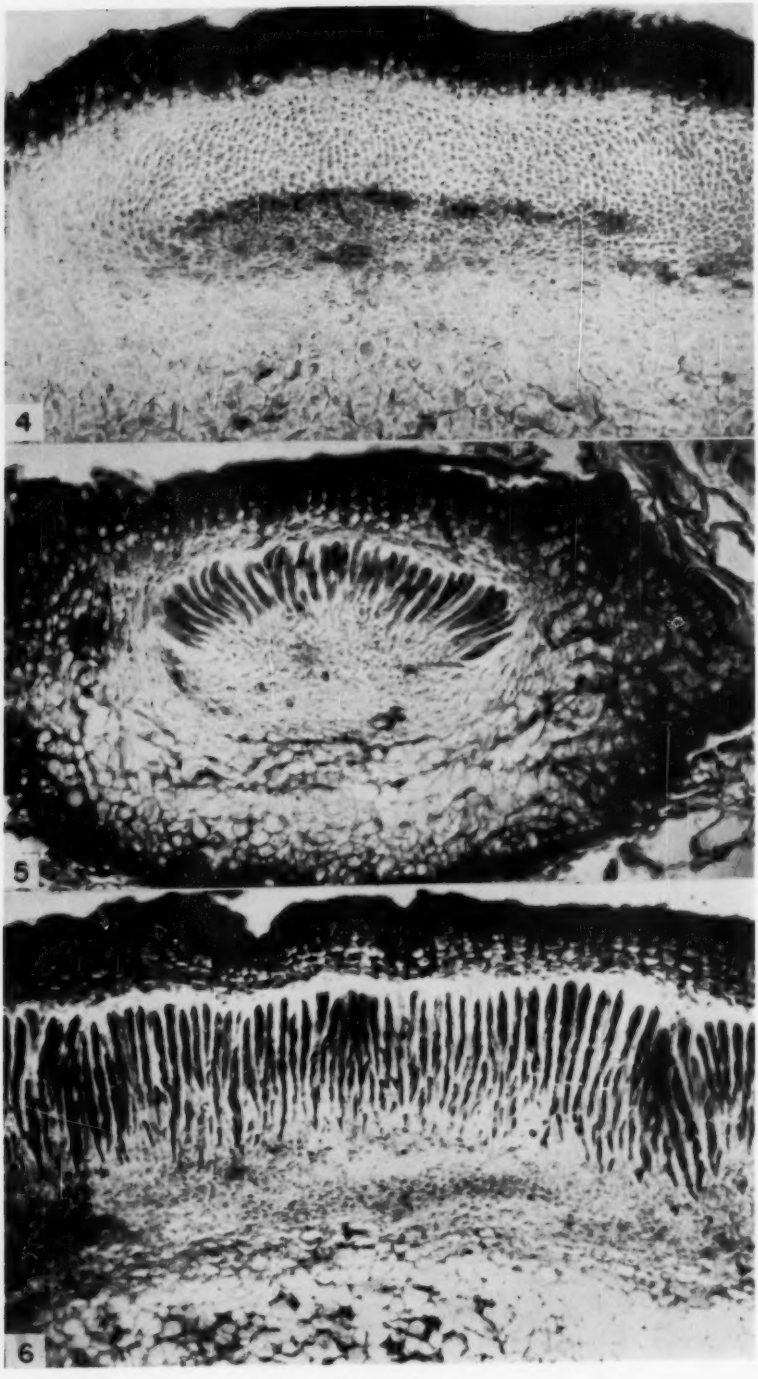
FIGS. 1–3.

A representative series of the permanent preparations on which this study is based has been deposited in the Herbarium of the Georgia Experiment Station. The section from which each illustration in this paper was made is indicated in the legends to the figures by a mixed number. The numeral refers to the slide number. The numerator of the fraction indicates the row of sections counted from top to bottom; the denominator indicates the number of the section counted from left to right when the slide is placed on the microscope with the label to the left.

MORPHOLOGY

The first evidence of development of *Dothiora schizospora* on stems cut in late winter and presumably naturally inoculated by the first of April was provided by the appearance of tiny pimples in the bark in early June. Sections of this material showed a mycelium of hyaline to brown, septate hyphae occupying and disrupting the inner layers of the bark. At points represented by the pimples observed macroscopically the hyphae had aggregated and produced cushion-shaped stromata, raising and rupturing the phellem of the host (FIG. 2). The stroma was composed of large, hyaline, polygonal, uninucleate cells. These cells often showed a tendency toward an arrangement in vertical columns. This did not appear to result from the lateral fusion of upward growing hyphae. The arrangement more nearly approached that of rows of tracheids formed from a cambium as seen in cross sections of stems of woody plants. Although no definite meristematic zone across the top of the stroma could be distinguished, it seems that the stromal tissue might properly be considered a true parenchyma. Cells at the periphery of the stroma and in a layer across the truncated apex became thick-walled and dark-colored. Above this zone at the apex was an outer layer of thin-walled, empty cells reminiscent of the root cap tissue in seed plants. Ascogonia provided with emergent trichogynes developed in the central region of the stroma (FIG. 2, 11), and microconidial locules producing bacilliform microconidia formed in the margins of the ascostromata or in separate stromata (FIG. 1). At this stage of its development the ascostroma of *Dothiora schizospora* showed a remarkable resemblance to the young ascostroma of *Dothidea collecta* (Schw.) Ell. & Ev. (= *Dothidea puccinioides* Fr. fide Loeffler (2)) as illustrated by

FIGS. 1–3. *Dothiora schizospora* (× 340). FIG. 1. Section of mature microconidial locule, slide No. 8-5/7. FIG. 2. Section of young ascostroma containing several ascogonia, slide No. 7-4/8. FIG. 3. Section of ascostroma with ascogonia producing ascogenous hyphae accompanied by early stages in the differentiation of the centrum, slide No. 10-3/20.



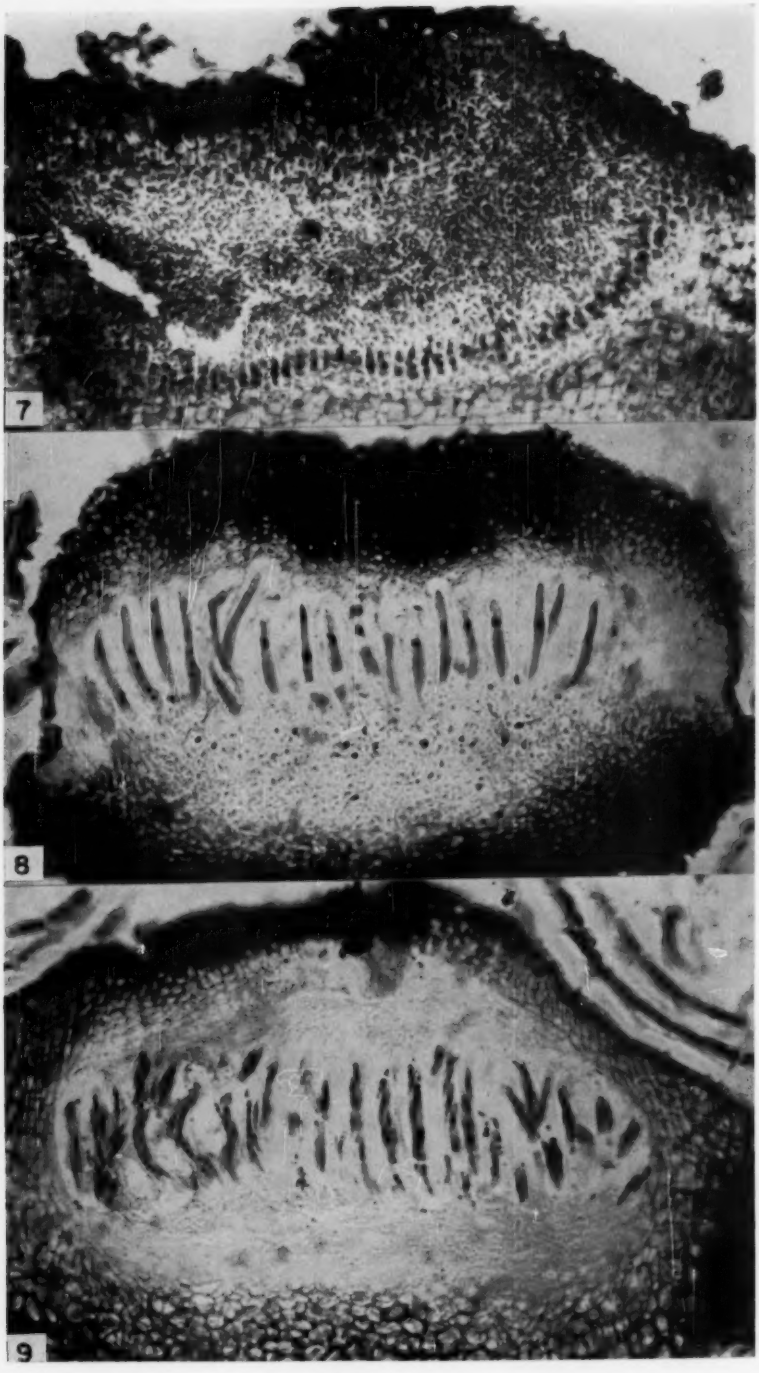
FIGS. 4–6.

Luttrell (3). From this stage onward, however, its development followed a divergent course. Shortly after the formation of ascogonia, the outlines of a single, broad, continuous locule appeared in the central region of the stroma; while the peripheral layers of dark, thick-walled cells increased in thickness and in density. The locule was filled with smaller, delicately-walled parenchyma cells with a denser protoplasm. A layer of deeply-staining ascogenous cells extended entirely across the base of the locule (FIG. 4). In November these cells produced a continuous palisade of young asci which pushed upward into the centrum parenchyma (FIG. 5). The asci produced mature ascospores by the end of February or in early March (FIG. 6). At maturity the ascocarps appeared as black, orbicular or irregularly oblong cushions protruding from the ruptured bark of the host stem. They measured 0.2–1.5 × 0.2–0.7 mm. The stromal tissue above the locule was longitudinally or irregularly split and pushed back to expose a dirty white to pale tan, gelatinous disc of asci. When moist, the ascocarp resembled a crude apothecium; when dry, the ruptured lips of the stroma closed over the disc of asci.

Development of the conidial stage paralleled the development of the ascigerous stage, the conidia maturing in late winter. The conidia were borne in large, lenticular locules occupying stromata indistinguishable from the ascostromata.

Microconidial locules.—The microconidial locules were occasionally formed in the margins of the ascostromata, but they usually occupied smaller separate stromata as shown in FIG. 1. In longitudinal sections such a stroma often appeared elongated and contained a linear series of similar locules which were more or less confluent. In the entire apical region of the stroma the large parenchyma cells became subdivided into numerous small, deeply-staining, uninucleate microconidial mother cells which then produced microconidia by budding. The microconidial mother cells arose in groups surrounded by a common cell wall (FIG. 10) and appeared to be produced by the internal division of the protoplasts of the large stromal parenchyma cells as described in *Dothidea puccinioides* (3). Division of the protoplast probably was accompanied by cell wall formation, but no cell walls delimiting the microconidial mother cells could be demonstrated. The angular shape and apparently

FIGS. 4–6. *Dothiora schizospora* (× 340). FIG. 4. Section of ascostroma with differentiated parenchymatous centrum and basal layer of ascogenous cells, slide No. 19-4/1. FIG. 5. Section of ascigerous locule filled with young, uninucleate asci, slide No. 31-5/8. FIG. 6. Longitudinal section of mature ascocarp containing a single broad locule filled with a palisade of aparaphysate asci, slide No. 49-3/13.



FIGS. 7–9.

loose arrangement of the microconidial mother cells shown in the figures undoubtedly resulted from shrinkage incident to fixation and preparation of the mounts. Disintegration of the exhausted mother cells produced a cavity filled with microconidia. Labyrinthine lobes of this cavity extended downward into the stroma as the formation of mother cells and microconidia continued. At the apex of the cavity the overlying layers of stromal cells opened by a pore or cleft through which the microconidia were extruded (FIG. 1, 10).

Ascogonia.—The ascogonia developed in the hyaline parenchyma toward the base of the stroma and were irregularly distributed, singly or in small clusters (FIG. 2, 11). They could be distinguished by their more deeply staining cytoplasm and more conspicuous nuclei. Like the surrounding stromal cells they were uninucleate. From each ascogonium a filamentous trichogyne extended upward between the stromal cells and projected slightly beyond the external layer of thin-walled, empty cells capping the stroma at this stage. The trichogynes contained several small nuclei and probably were divided into elongated cells, although septations could not be clearly discerned except at the juncture with the ascogonial cells. The protruding tips were swollen and were surrounded with thick, hyaline walls. The most nearly complete ascogonial apparatus observed is shown in FIG. 11 which illustrates three globose ascogonia, fragments of two trichogynes reaching two-thirds of the way to the surface, and two protruding trichogyne tips. Microconidia were found on the surface of the stroma and lying against the trichogyne tips. Although genetic evidence is lacking and the cytological evidence is incomplete, it is assumed that these ascogonia and the microconidia constitute the sexual mechanism in *Dothiora schizospora.*

Ascigerous locules.—Presumably following fertilization by the microconidia, the ascogonia became multinucleate (FIG. 12). At about this time the cells of the central parenchyma above the ascogonia became smaller and stained more deeply (FIG. 3). This development progressed until it involved a disc-shaped area extending across the entire central region of the ascostroma (FIG. 4). This constituted the sterile tissue of the centrum occupying the region that would become the single broad locule of the mature ascocarp. There was no evidence of the internal division of the larger stromal cells to form the centrum paren-

FIGS. 7–9. *Dothiora* spp. (× 340). FIG. 7. Section of mature conidial locule of *D. schizospora,* slide No. 49-3/1. FIG. 8. Section of ascocarp of *D. sphaeroides* with elongated asci undergoing meiotic divisions, slide No. 54-5/9. FIG. 9. Section of mature ascocarp of *D. sorbi* showing single broad locule filled with a palisade of aparaphysate asci, slide No. 51-1/12.

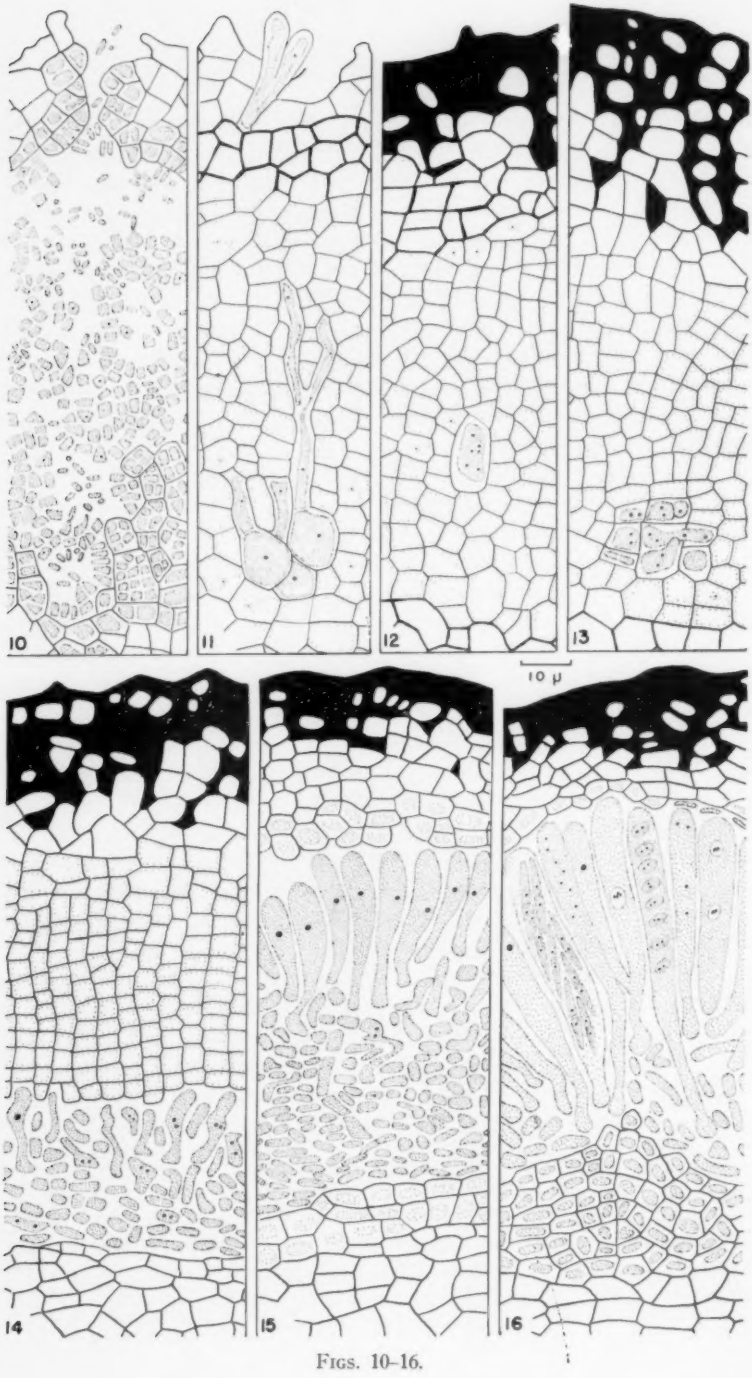
chyma as described in *Dothidea puccinioides* (3), but this seems to be the only explanation for the reduction in size of these cells. Concurrently, the multinucleate ascogonia divided into several binucleate segments. A few rare sections such as the one illustrated in FIG. 13 provided some evidence that these binucleate segments produce tubular outgrowths which spread laterally between the stromal cells and become segmented to form binucleate ascogenous cells. In any event, the floor of the locule became covered with a thick layer of deeply-staining ascogenous cells (FIG. 4, 14). The chief basis for interpreting these cells as ascogenous cells was their staining properties. Their nuclear condition was apparent only in occasional cells. The frequency of such cells is exaggerated in FIG. 14 because this illustration is a composite to the extent that drawings of several ascogenous cells clearly showing the nuclei were taken from other portions of the section and inserted in this figure. The ascogenous cells extended upward and formed binucleate asci. There was no evidence of crosier formation, but negative evidence on this point from sectioned material is not conclusive. Slightly larger asci contained a single conspicuous nucleus, presumably resulting from the fusion of the two nuclei originally present (FIG. 14). The uninucleate asci elongated to form a close palisade, pushing upward as a group into the delicate centrum parenchyma (FIG. 5, 15). This parenchyma was crushed and disintegrated completely. There was no trace of interascicular tissue of any sort at any stage in the development of the asci.

The ascus remained uninucleate until it had reached nearly mature size. The large fusion nucleus then rapidly passed through three successive divisions to produce eight nuclei around which eight ascospores were delimited. Asci in the uninucleate, 4-nucleate, and 8-nucleate condition; with metaphase figures of the first and second divisions; and in the process of ascospore formation are shown in FIG. 16 which is a composite in that it includes asci in various stages assembled from several successive sections of the ascocarp illustrated. Four poorly distinguishable chromatic bodies appeared in each metaphase plate. When first delimited, the ascospores were ovoid or ellipsoid and were arranged in linear sequence. Each contained two nuclei, indicating a nuclear division almost simultaneous with the formation of spore membranes. The ascospores elongated to their typical fusoid shape and were divided by a primary septum into two uninucleate cells. Further nuclear divisions and the insertion of additional septa produced the mature spores. The elongating spores slipped past one another into an irregularly tristichous arrangement.

During the development of the asci, the peripheral layer of dark thick-walled stromal cells became progressively thicker and more opaque. The centrum parenchyma disintegrated, leaving a broad, disc-shaped locule filled with a palisade of asci arising from a thick placenta of ascogenous cells. When a long, narrow ascocarp such as that shown in FIG. 6 was examined in cross section, the locule appeared nearly spherical with the asci radiating from a hemispherical basal placenta (FIG. 16). In such sections the locule appeared nearly identical with a mature locule of *Dothidea puccinioides* (**3**). There was no evidence of any opening mechanism in the stromal layer covering the locule, and it is assumed that the irregular splitting of this layer at maturity results simply from the pressure of the expanding asci.

Asci.—Mature asci were subcylindric or clavate and tapered slightly toward the base into a short stalk (FIG. 17). They measured 70–119 × 11–15 μ. They were typical of the bitunicate type, thick-walled when young but appearing thin-walled when fully expanded prior to dehiscence. At dehiscence the thin outer wall split at the apex, and the inner wall expanded until the length of the ascus was doubled or tripled (FIG. 17). At the apex of the expanded inner wall was a small papilla which apparently contained an elastic pore. The uppermost ascospore moved into this pore. Usually the spore was caught at one of its constrictions and hung momentarily in the pore before being violently shot away. The remaining spores went through a similar process of ejaculation in succession. In empty asci or in expanded asci that lost turgor before spore discharge the inner wall thickened greatly, and the thin outer wall formed a wrinkled collar constricting the middle of the tubular inner wall. *Dothiora schizospora* furnished exceptionally favorable material for observation of spore discharge because the entire process occurred readily in crushed mounts in water.

Mature ascospores (FIG. 18) were uniformly hyaline but were variable in size and septation. They were fusiform, 20–44 × 4–6 μ (mean 27.3 × 4.7 μ) in total dimensions, and 1–9-septate with occasionally longitudinal septa in one or two of the central cells. They were slightly constricted at all septa but at the primary septum were nearly cut in half by a deep constriction. The anterior part was larger, 10.6–22.8 × 4.4–6.2 μ, and was 0–6-septate; the posterior part measured 9.9–21.3 × 3.0–5.8 μ and was 0–3-septate. When the spores were discharged onto agar plates, they were scattered individually in the irregular patterns characteristic of this type of successive ascospore ejaculation, and each spore broke at the deep median constriction, the two halves lying side by side on the agar surface (FIG. 19).



FIGS. 10–16.

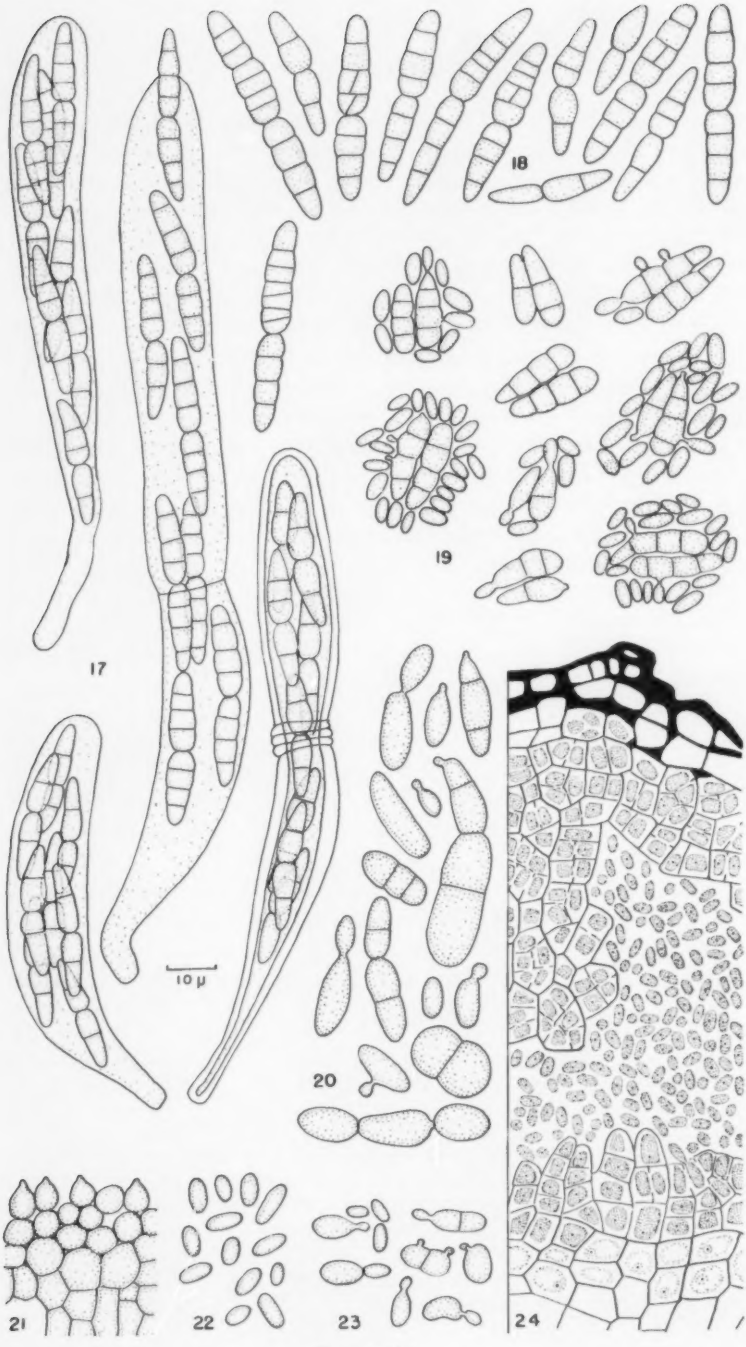
Conidia.—The conidia formed in stromata identical with the ascostromata, and occasionally a conidial locule and an ascigerous locule were found occupying a single stroma. They developed in broad lenticular cavities (FIG. 7) lined with small ovoid cells measuring 4.5–6 μ in diameter (FIG. 21). At the apex of each cell was a papilla from which the conidia were produced as buds. The conidia therefore are properly blastospores. They (FIG. 22) were 1-celled, hyaline, and ovoid or usually ellipsoid and measured 4–9 × 2–4 μ (mean 6.5 × 3.3 μ).

As observed in stained sections, the process of conidium-formation appeared to be the same as in the formation of microconidia (FIG. 24). The protoplasts of the large stromal cells divided internally to form two or more conidial mother cells which produced conidia and disintegrated, leaving a cavity filled with conidia. The angular, shrunken appearance of the mother cells here was obviously the result of fixation as was indicated by a comparison with their appearance in freehand sections of fresh material mounted in water (FIG. 21). It was also apparent that division of the protoplasts of the original stromal cells was accompanied by the formation of septa. The conidial mother cells, therefore, were produced from the stromal cells by fission rather than by free cell formation.

The conidial locules developed concurrently with the ascigerous locules, the conidia maturing at about the same time as the ascospores or slightly earlier. The factors determining the course of differentiation of the stromata are unknown, although it is possible that conidium-formation proceeds in stromata in which fertilization of the ascogonia fails to occur.

Spore germination and growth in culture.—On media such as 2% malt extract agar and 20% V-8 Juice agar ascospores germinated by the indiscriminant production of buds from the tips and sides. Each pair of ascospore halves soon was surrounded by a mass of hyaline, ellipsoidal cells resembling the conidia (FIG. 19). The conidia ger-

FIGS. 10–16. *Dothiora schizospora* (all to scale in FIG. 16). FIG. 10. Section of microconidial locule, slide No. 8-5/7. FIG. 11. Group of three uninucleate ascogonia and fragments of trichogynes in section of young ascostroma, slide No. 9-1/15. FIG. 12. Multinucleate ascogonium after fertilization, slide No. 10-3/21. FIG. 13. Fragmentation of ascogonia into binucleate cells which produce ascogenous hyphae, slide No. 10-3/20. FIG. 14. Section of ascostroma showing the sterile parenchymatous tissue of the centrum and basal layer of ascogenous cells producing asci, slide No. 19-4/1. FIG. 15. Section of ascigerous locule with uninucleate asci expanding into the disintegrating centrum parenchyma, slide No. 33-3/7. FIG. 16. Section of nearly mature ascocarp with asci in stages of nuclear divisions and ascospore delimitation, slide No. 5-2/15.



FIGS. 17–24.

minated in the same fashion (FIG. 23), often swelling and becoming 1-septate in the process.

Cultures from both sources were identical, forming cream-colored, yeast-like colonies that soon turned black. Cells in culture continued to reproduce by budding (FIG. 20). They were variable in size and shape and were often 1–2-septate. Some clung together in short chains. Older cells became dark brown. When the yeast-like cultures were transferred to sterilized *Symphoricarpos* stems, a normal gray mycelium developed.

The connection between the conidial and ascigerous stages is based on the identity of cultures from ascospores and from conidia; on the similarity of the conidial and ascigerous stromata, both types of locules occasionally being produced in the same stroma; and on the fact that, when sterilized stems of *Symphoricarpos* were inoculated with an ascospore culture and placed out of doors at Experiment, Georgia, where neither the host nor the fungus occurs naturally, conidial stromata developed on the stems.

DISCUSSION

The Loculoascomycetes with Discomycete-like ascocarps which von Arx and Müller (**1**, **7**) assembled in the order Dothiorales resemble one another in the production of a discoid, ellipsoid, or linear ascostroma containing a single indefinitely limited locule filled with a continuous palisade of asci which are exposed at maturity by the rupture of the overlying stromal tissue. However, in their fundamental patterns of development two distinct types may be recognized.

In *Glonium stellatum* Mühl. ex Fr., which appears representative of the Hysteriaceae, the locule is formed, at least in part, by the growth of pseudoparaphyses; and the mature centrum is composed of a palisade of asci interspersed with persistent pseudoparaphyses (**5**). This represents a variant of the Pleospora Developmental Type (**4**), *Glonium* differing from a typical representative of this type such as *Thyridium* in that the ascostroma is occupied by a single, flat, continuous locule rather

FIGS. 17–24. *Dothiora schizospora* (all to scale in FIG. 17). FIG. 17. Mature asci, expanded ascus in process of ascospore ejaculation, and collapsed ascus showing thickened inner wall and thin outer wall forming wrinkled collar. FIG. 18. Mature ascospores. FIG. 19. Discharged, fragmented ascospores germinating by budding. FIG. 20. Budding cells from yeast-like culture on agar. FIG. 21. Freehand section of fresh material showing cells lining conidial locule. FIG. 22. Mature conidia. FIG. 23. Conidia germinating by budding. FIG. 24. Section of conidial locule, slide No. 49-3/1.

than by several spherical locules. The relationships of the Hysteriaceae, therefore, lie with the Pleosporaceae (Pleosporales), although there may be sufficient justification for retaining the Hysteriaceae in a separate order, the Hysteriales (**5, 6**).

In *Dothiora schizospora* the locule is formed by the disintegration of a parenchymatous centrum in which the aparaphysate asci arise in a single large cluster. From study of sections of the type species of *Dothiora, D. sorbi* (Wahlbg.) Rehm (FIG. 9) and from the illustration of this species by Müller and von Arx (**7**) it seems probable that *D. sorbi* is essentially similar in structure to *D. schizospora*. Sections of immature ascocarps of *D. sphaeroides* Pers. ex Fr. (FIG. 8) likewise revealed a single broad layer of aparaphysate asci developing within the parenchymatous centrum of a uniloculate ascostroma. The pattern of development in *Dothiora,* therefore, bears much the same relationship to the Dothidea Developmental Type (**4**) as does that of *Glonium* to the Pleospora Developmental Type. The striking parallels noted in the development of *Dothiora schizospora* and of *Dothidea puccinioides* (**3**) afford further evidence of the close structural relationship between these two genera. Accordingly, the Dothioraceae is associated with the Dothideaceae in the order Dothideales, as has been suggested previously (**5, 6**), and the Dothiorales of Müller and von Arx (**7**) is considered a synonym of the Dothideales.

SUMMARY

Dothiora schizospora sp. nov. was found on dead stems of *Symphoricarpos orbiculatus* Moench. in Missouri. Its most striking specific character is the production of fusoid, phragmosporous or muriform ascospores which are so deeply constricted at the primary septum that they readily separate into halves. The asci are bitunicate. The ascocarp is a parenchymatous, orbicular or oblong ascostroma containing a single, broad, flat locule filled with a continuous palisade of aparaphysate asci which are exposed by the rupture of the overlying stromal tissue and then resemble the disc of an apothecium. The centrum is composed of a parenchymatous tissue which disintegrates as the asci mature. The sexual apparatus consists of globular ascogonia with filamentous trichogynes projecting above the surface of the stroma and microconidia produced in locules in the same or separate stromata. A *Dothichiza* conidial stage is present. The Dothioraceae are classified alongside of the Dothideaceae in the order Dothideales of the subclass Loculoascomycetes.

GEORGIA EXPERIMENT STATION
EXPERIMENT, GEORGIA

LITERATURE CITED

1. **Arx, J. A. von, and E. Müller.** 1954. Die Gattungen der amerosporen Pyrenomyceten. Beitr. Kryptogamenflora Schweiz **11** (1): 1–434.
2. **Loeffler, W.** 1957. Untersuchungen über die Ascomyceten-Gattung *Dothidea* Fr. Phytopath. Zeitschr. **30**: 349–386.
3. **Luttrell, E. S.** 1951. The morphology of *Dothidea collecta*. Amer. Jour. Bot. **38**: 460–471.
4. **Luttrell, E. S.** 1951. Taxonomy of the Pyrenomycetes. Univ. Missouri Studies **24** (3): 1–120.
5. **Luttrell, E. S.** 1953. Development of the ascocarp in *Glonium stellatum*. Amer. Jour. Bot. **40**: 626–633.
6. **Luttrell, E. S.** 1955. The ascostromatic Ascomycetes. Mycologia **47**: 511–532.
7. **Müller, E., and J. A. von Arx.** 1950. Einige Aspekte zur Systematik pseudophärialer Ascomyceten. Ber. Schweiz. Bot. Ges. **60**: 329–397.
8. **Nannfeldt, J. A.** 1932. Studien über die Morphologie und Systematik der nicht-lichenisierten inoperculaten Discomyceten. Nova Acta Regiae Soc. Scien. Upsaliensis, Ser. IV. **8**: 1–368.

FACTORS AFFECTING THE PRODUCTION OF CAROTENE BY CHOANEPHORA CUCURBITARUM [1, 2]

F. S. CHU [3] AND VIRGIL GREENE LILLY

(WITH 19 FIGURES)

Goodwin (**1952**, **1954**, **1955**, and **1958**) has reviewed the literature on the synthesis of carotene by fungi. Relatively few species have been used for intensive study of carotenogenesis: *Phycomyces blakesleeanus*, *Neurospora crassa*, *Mucor hiemalis*, and *Blakeslea trispora*. Barnett et al. (**1956**) found that mixed + and − cultures of *Choanephora cucurbitarum* produced much more carotene than cultures of either sex singly. An increase in carotenogenesis also occurs with mixed + and − cultures of other species of Choanephoraceae (Hesseltine and Anderson, **1957**; Anderson et al., **1958**). Wolf (**1917**) found numerous yellow oil droplets in immature zygospores and Barnett et al. (**1956**) showed that the principal pigment was *beta*-carotene.

The effects of nutritional and environmental factors on growth, glucose utilization, pH values, and the synthesis of carotene are reported in this paper.

MATERIALS AND METHODS

Two isolates (+ and −) of *Choanephora cucurbitarum* (Berk. and Rav.) Thaxter were used in this work. They were taken from the stock culture collection of the Department of Plant Pathology, Bacteriology and Entomology and are the same isolates used in previous studies reported from this laboratory (Barnett and Lilly, **1950**, **1955**, **1956** and Barnett et al., **1956**).

The asexual spores used as inoculum were produced on plates of glucose yeast extract agar medium. Spores from the + and the − isolates were collected separately and suspended in sterile distilled water.

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[2] It is a pleasure to acknowledge a grant from the Rockefeller Foundation which has in part supported this work.

[3] Based on the M.S. Thesis of the senior author, present address: 515 S. Fifth Street, Columbia, Missouri.

Each tube of spore suspension was tested for contamination before storing at −5° C.

The basal medium had the following composition: D-glucose, 20 g; L-glutamic acid, 2 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; Fe(III) and Zn(II), 0.2 mg each; Mn (II), 0.1 mg; Ca(II), 10 mg; thiamine hydrochloride, 100 μg; and distilled water to make one liter. The pH of the medium was adjusted to 6.0 before autoclaving. Variations in the medium are described in connection with individual experiments. The culture vessels were 250-ml Pyrex Erlenmeyer flasks. Twenty-five ml aliquots were machine pipetted into the flasks which were capped with 50-ml Pyrex beakers. Media were autoclaved 15 minutes at 121° C. When single isolates were cultured, one drop of spore suspension was added to each flask of medium; when mixed cultures were desired, one drop each of + and − spores was added to each flask.

Unless otherwise noted, the cultures were incubated at 28° C in a room which was illuminated 12 hours daily with fluorescent lamps. The cultures were not disturbed until harvested. Harvests were made at intervals of one or two days.

The mycelium from two flasks was collected individually, washed with distilled water, dried overnight, and weighed on an analytical balance. The pH values of the culture filtrates were determined with a Cambridge pH meter. The residual glucose in the culture filtrates was determined by a modification of the Folin-Wu method (Keller, **1951**). Lipids were determined by exhaustively extracting the dry pellets of mycelium with diethyl ether in a Soxhlet apparatus. Carotene was extracted from two additional freshly harvested mycelial mats using peroxide-free diethyl ether (Garton et al., **1951**) and the carotene content measured with a Beckman model DU spectrophotometer at 452 mμ. The carotene content (as *beta*-carotene) is reported as μg/culture, or in some instances as mg/g dry mycelium.

Most of the experiments were done two or more times. The results were similar and usually agreed within ±10 per cent. Data from individual experiments are reported in Experimental Results.

EXPERIMENTAL RESULTS

The effects of concentration of glucose, carbon sources, nitrogen sources, acetate, *beta*-ionone, temperature, light, and initial pH on growth, carotenogenesis, and other processes were studied.

Concentration of glucose. Five concentrations of glucose (20, 40, 60, 80, and 100 g/l) were used in a series of experiments. Separate + and − cultures as well as mixed + and − cultures were studied.

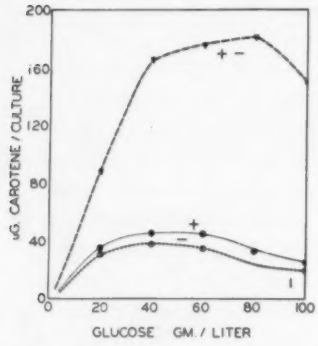


FIG. 1. Maximum yields of carotene by +, −, and mixed + and − cultures when the glucose concentration in the glutamic acid medium varied between 20 and 100 g/l.

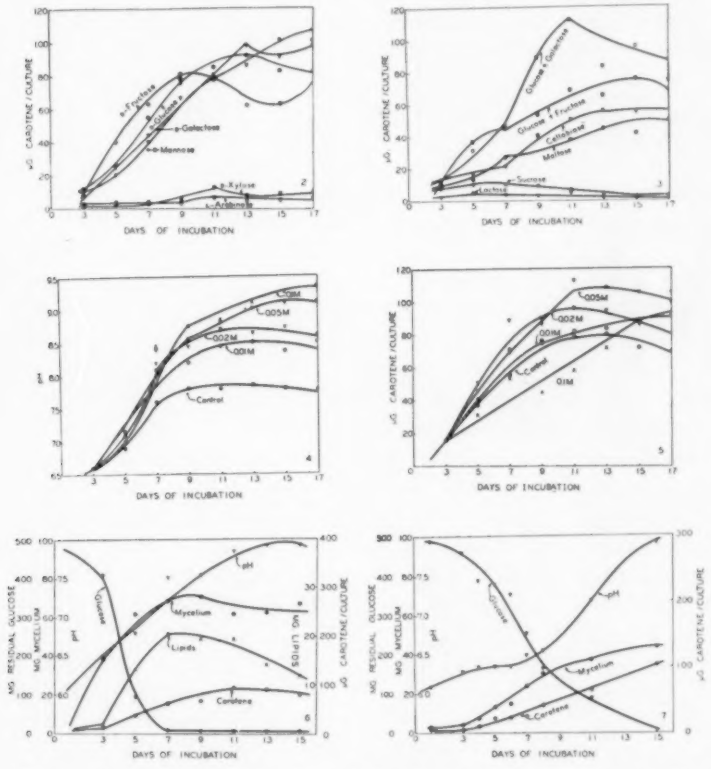
The weights of mycelium produced by the three types of cultures did not differ significantly. The weight of mycelium increased as the concentration of glucose was increased from 20 to 60 g/l; slightly less mycelium was produced on media containing 80 and 100 g of glucose than in the medium containing 60 g. The time required for the mycelium to attain maximum weight increased as the concentration of glucose increased from 20 to 60 g/l. The utilization of glucose was essentially complete on the day maximum weight was attained. The utilization of glucose was not complete in 18 days when the media contained 80 and 100 g of glucose; the residual glucose was about 450 and 840 mg/25 ml, respectively.

The + isolate produced slightly more carotene than the − isolate. Mixed + and − cultures produced 3 to 5 times as much carotene as either the + or − cultures grown separately. Data for the maximum carotene yields are shown in FIG. 1. Only a slight increase in carotene production by either the + or − isolate occurred when the glucose concentration was increased above 20 g/l. There was a decided increase in carotene synthesis by mixed + and − cultures when the glucose concentration was increased from 20 to 60 g, and a slight increase when the glucose concentration was raised to 60 and 80 g.

The lipid content of the mycelium increased as the glucose concentration was increased from 20 to 100 g/l. The lipid content continued to increase with the time of incubation, or until the glucose was exhausted. The mycelium contained 31.0, 35.8, 46.7 and 49.2 per cent lipids (maximum values) when the medium contained 20, 60, 80, and

100 g of glucose, respectively. A yellow oil, which floated on the surface of the medium, was liberated from the mycelium when the media contained 60 to 100 g of glucose. *Choanephora cucurbitarium*, like other fungi, synthesizes more lipid material when the carbohydrate concentration is high.

When the basal medium was used the carotene content of the mycelium increased for about 5 days after the glucose was exhausted and the increase in weight ceased. The lipid content decreased after the



FIGS. 2-7. FIG. 2 (upper left). Production of carotene on monosaccharide media. FIG. 3 (upper right). Production of carotene on mixed monosaccharide, and disaccharide media. FIG. 4 (center left). Effects of added potassium acetate on pH values. FIG. 5 (center right). Effects of added potassium acetate on the production of carotene. FIG. 6 (lower left). Control for FIGS. 7–11. FIG. 7 (lower right). Effects of adding 5 μl of *beta*-ionone to 1-day-old cultures. Mixed + and − cultures were used in all experiments.

glucose was utilized (FIG. 6). These data suggest that lipids may serve as carbon and energy sources for the synthesis of carotene.

Carbon sources. The following carbon sources were tested: D-glucose, D-fructose, D-galactose, D-mannose, D-xylose, L-arabinose, lactose, sucrose, maltose, cellobiose, raffinose, starch, cellulose, and molasses (Brer Rabbit, Gold Label). Molasses was used at the rate of 30 ml/l, while the other carbohydrates were used in amounts that supplied 8 g C/l. Equimolecular mixtures of glucose and fructose, and glucose and galactose were used in some experiments. The hexoses were the best carbon sources for carotenogenesis. The maximum weights of mycelium produced on glucose, fructose, mannose, and galactose media were 75.6 (7 days), 81.5 (9 days), 91.3 (7 days) and 102.5 (9 days) mg, respectively. Chromatographic examination of the filtrates showed that utilization of these sugars was essentially complete by the time the maximum weights of mycelium were produced. Some of the data on carotene production obtained when different sugars and mixtures of sugars were used are given in FIGS. 2 and 3. The carotene content of the cultures grown on glucose, mannose, and galactose media continued to increase after the sugars were utilized and the weights began to decrease. Growth in the xylose and arabinose media was slow. The mycelium from the xylose medium weighed 88.3 mg (19 days), and 77.9 mg (19 days) from the arabinose medium. Chromatographic examination showed that the pentoses were present on the seventeenth day. The yields of carotene on the pentose media were low (FIG. 2).

The production of carotene on the medium containing equal amounts of glucose and galactose was more rapid than when galactose alone was used; however, less mycelium was produced on the mixture than on galactose alone. An equimolecular mixture of glucose and fructose was no better than the individual sugars for the production of carotene. Four disaccharides were tested. Cellobiose was an excellent carbon source for growth (133.6 mg, 11 days); maltose was less satisfactory (87.2 mg, 9 days). Chromatographic examination of the filtrates showed that cellobiose was completely hydrolyzed by the ninth day and maltose by the seventh. Only a trace of growth resulted when sucrose, lactose, and raffinose were used. Slightly more carotene was produced on cellobiose than on maltose media. These disaccharides yield glucose on hydrolysis, yet less carotene was produced on these sugars than on glucose. *Choanephora cucurbitarum* grew on soluble starch (89.3 mg, 17 days) and produced some carotene (49.5 μg, 11 days). Some growth occurred on cellulose (Whatman filter paper) but only a trace of carotene was produced.

Molasses (30 ml/l) was a good carbon source for growth and only fair for carotenogenesis. Growth was complete in 3 days (FIG. 12). The yield of carotene in various experiments was about equal to that obtained with the mixed glucose and fructose medium (FIG. 3). When molasses was analyzed by paper chromatography, raffinose, sucrose, glucose, and fructose were identified. The reducing sugar content of the molasses medium was 10.6 g/l (calculated as glucose). By the third day of incubation, only traces of glucose and fructose remained in the medium. The concentration of raffinose and sucrose did not appear to change within 19 days. Additional experiments with molasses are reported in the section on *beta*-ionone.

Nitrogen sources. Fourteen sources of nitrogen were tested. *Choanephora cucurbitarum* did not utilize nitrate nitrogen or glycine. The single nitrogen sources were used in amounts which supplied 0.19 g N/l (equivalent to 2 g of glutamic acid). The complex natural nitrogen sources were used at the rate of 2 g/l. The maximum yields of carotene are summarized in TABLE 1. See also FIGS. 14 and 16.

Glutamic acid was the best simple nitrogen source tested. Enzymatic casein hydrolysate and yeast extract were good natural sources of nitrogen. The closely related compounds asparagine and aspartic acid differed widely in value as nitrogen sources. The failure of *Choanephora cucurbitarum* to synthesize more than a trace of carotene on the ammonium sulfate medium may be attributed in part to low pH. Glucose utilization was slow in this medium; 263 mg/25 ml remained after 17 days of incubation.

Effects of acetate. Media containing acetate as the sole source of carbon have been used to study carotenogenesis in *Phycomyces blakesleeanus* (Schopfer and Grob, **1952**) and *Mucor hiemalis* (Grob and Bütler, **1954**).

Choanephora cucurbitarium grew slowly in an acetate-ammonium sulfate medium. In the experiments below, potassium acetate was added to cultures growing in the basal glucose-glutamic acid medium. Sterile potassium acetate solutions (0.5 ml/25 ml) were added to four sets of 3-day-old cultures. At the time acetate was added, the concentrations were 0.01, 0.02, 0.05, and 0.1 M (24.5, 49, 123, and 245 mg/25 ml, respectively). Neither growth nor glucose utilization was appreciably affected by these additions of potassium acetate. However, these additions caused the pH values of the culture media to increase from one to two units (FIG. 4). Carotene synthesis was increased by the addition of the three lowest concentrations of potassium acetate, and decreased initially by the addition of the highest concentration (FIG. 5). After

TABLE I

MAXIMUM YIELDS OF CAROTENE (AVERAGE OF 2 CULTURES) WHEN DIFFERENT SOURCES OF NITROGEN WERE USED IN THE BASAL MEDIUM. TWENTY G OF GLUCOSE/L WAS USED IN ALL MEDIA

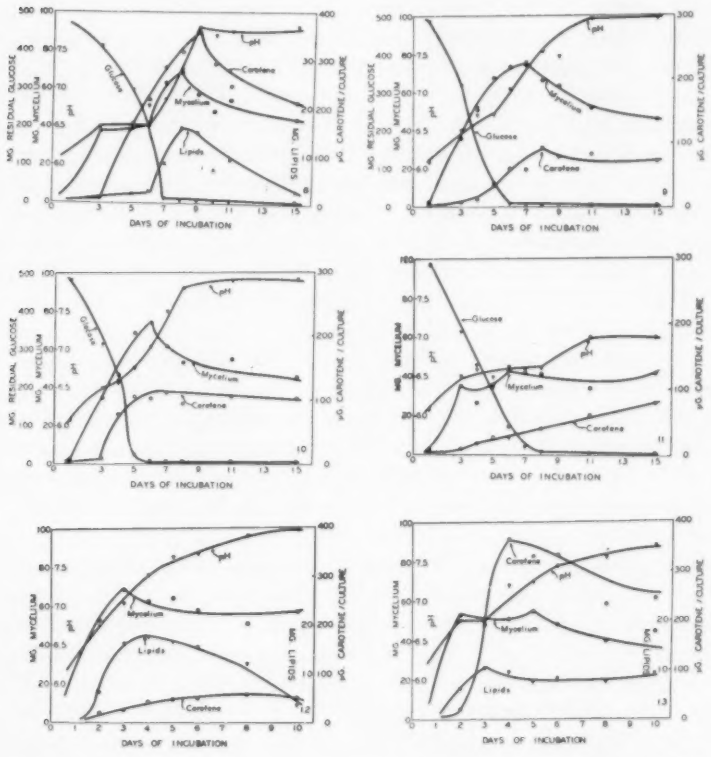
Nitrogen sources	Day of maximum yield	Mycelium mg	pH values	Residual glucose/ culture mg	Carotene	
					μg/culture	mg/g
L-glutamic acid	15	62.0	7.85	3	89	1.44
Ammonium sulfate	3	29.8	2.63	415	7	0.24
Urea	17	52.0	6.14	1	35	0.67
L-asparagine	15	53.2	6.94	2	46	0.87
DL-valine	17	48.5	6.48	5	39	0.81
L-leucine	13	43.6	6.90	3	39	0.89
L-arginine	15	53.9	5.20	3	56	1.04
L-aspartic acid	19	36.8	6.60	7	17	0.47
L-proline	19	43.6	6.90	3	39	0.89
Yeast extract	9	70.8	6.27	5	75	1.06
Dry yeast cells	13	59.3	5.65	4	53.0	0.89
Casein hydrolysate (Enzymatic)	13	76.1	5.20	4	69	0.91

about the thirteenth day, more carotene was found in the cultures to which the highest concentration of acetate was added than in the control cultures.

It would appear that either acetate was used as a carbon source for the synthesis of carotene, or that in the presence of acetate more of some constituent, possibly lipids, was used for this purpose. The increase in pH values of the media receiving acetate suggests strongly that the acetate was utilized. Two experiments were carried out with radioactive acetate to test this assumption. A glutamic acid medium containing 60 g of glucose was used. In the first experiment, 12.2 mg of potassium acetate and 2 μc of sodium acetate-1-C^{14} were added to each flask in a set of 3-day-old cultures. Eighty-five per cent of the radioactivity disappeared from the medium within 24 hours. In the second experiment, acetate was added on the sixth day. Forty-six per cent of the radioactivity disappeared in 24 hours and 79 per cent within 48 hours. These results show that *C. cucurbitarum* utilized acetate under these conditions.

Effects of beta-ionone. MacKinney et al. (**1952**) discovered that the addition of minute amounts of *beta*-ionone to cultures of *Phycomyces blakesleeanus* increased the synthesis of *beta*-carotene. The *beta*-ionone effect has been repeatedly confirmed for *P. blakesleeanus* and other fungi. *beta*-Ionone appears to affect carotenogenesis by altering the metabolism rather than by serving as a precursor of *beta*-carotene (MacKinney et al., **1953**; Engel et al., **1953**; Lilly et al., **1960**). A detailed study of the effect of *beta*-ionone on *Choanephora cucurbitarum* was made.

1. Effect of quantity and time of addition of *beta*-ionone. The basal glucose-glutamic acid medium was used in the experiments reported in this section. From 1 to 10 μl of *beta*-ionone was added to cultures varying in age from 1 to 7 days. A 10 μl micro pipette was used to add *beta*-ionone to the surface of the cultures. Dilutions of *beta*-ionone in absolute ethanol were used when necessary. A control experiment showed that the addition of 10 μl of absolute ethanol had no effect on



FIGS. 8–13. FIG. 8 (upper left). Effects of adding 5 μl of *beta*-ionone to 3-day-old cultures. FIG. 9 (upper right). Effects of adding 5 μl of *beta*-ionone to 7-day-old cultures. FIG. 10 (center left). Effects of adding 1 μl of *beta*-ionone to 3-day-old cultures. FIG. 11 (center right). Effects of adding 10 μl of *beta*-ionone to 3-day-old cultures. FIG. 12 (lower left). Control, molasses-glutamic acid medium. FIG. 13 (lower right). Effects of adding 5 μl of *beta*-ionone to 2-day-old cultures grown on molasses-glutamic acid medium. Mixed + and − cultures were used in all experiments.

growth or carotenogenesis. Typical results are shown in FIGS. 6 to 11.

The effects of *beta*-ionone depend upon the amount added and the time of addition. The comparison of the results in FIGS. 6 to 9 reveals that the effects of adding 5 μl of *beta*-ionone change with the time of addition. When the cultures were 1 day old, the rate of growth, glucose utilization, and the normal elevation of pH values were severely depressed (FIG. 7). The early synthesis of carotene was first depressed but eventually the yield of carotene by the *beta*-ionone treated cultures slightly exceeded the yield of the control cultures (FIG. 6). When *beta*-ionone was added to 3-day-old cultures (FIG. 8), the rate of growth was decreased between the third and seventh day, but glucose utilization was about equal to that of the control cultures (FIG. 6). The yield of carotene in the treated cultures was about 5 times that of the control cultures. Lipid synthesis was less in the *beta*-ionone treated cultures than in the controls (FIGS. 6 and 8). When 7-day-old cultures were treated with 5 μl of *beta*-ionone (FIG. 9) only a slight increase in carotene synthesis resulted. The effects of adding 1, 5, and 10 μl of *beta*-ionone to 3-day-old cultures are seen by comparing FIGS. 6, 8, 10, and 11.

The greatest response of mixed + and − cultures to added *beta*-ionone was when about half of the maximum weight of mycelium is produced, and when zygospore initials are beginning to form (3 days, in the basal medium). The maturation of zygospores was delayed by the addition of *beta*-ionone. Mature zygospores were found in the control cultures by the fifth day. The addition of 5 μl of *beta*-ionone to 1- and 3-day-old cultures delayed maturation of zygospores until about the ninth day.

The effect of adding 5 μl of *beta*-ionone to 3-day-old cultures of + and − isolates was determined. On the fifteenth day the carotene content of the + controls was 20 and that of the *beta*-ionone treated cultures 31 μg/culture. The corresponding carotene values for the − sex were 15 and 21 μg/culture. While some increase in carotene production resulted when cultures of the + and − sexes singly were treated with *beta*-ionone, the increase was small. *beta*-Ionone does not replace the factors produced by the two sexes which stimulate carotenogenesis, but acts synergistically with them.

2. Molasses-glutamic acid medium. Molasses (30 ml/l) was used in place of glucose in these experiments. Five μl of *beta*-ionone was added to 2-day-old cultures since growth was exceedingly rapid on this medium. Some of the data are presented in FIGS. 12 and 13. Mixed + and − cultures grown on the molasses-glutamic acid medium and

treated with 5 μl of *beta*-ionone on the second day produced more carotene than on any other medium tried. The maximum yield was obtained on the fourth day and was in excess of 7 mg/g dry mycelium. This is less than the yield (9 mg/g dry mycelium) reported by Ciegler et al. (**1959a**) from mixed + and − cultures of *Blakeslea trispora* grown on a complex natural medium and treated with *beta*-ionone. A single isolate of *Phycomyces blakesleeanus* grown on a synthetic medium and treated with *beta*-ionone produced 4 mg/g of dry mycelium (Lilly et al., **1960**).

The surface of the *beta*-ionone treated cultures was red to brown instead of the usual yellow. A sample of mycelium treated with *beta*-ionone was extracted with hexane and the extinction measured at various wave lengths. The maximum absorption was at 452 mμ, indicating that the principal pigment was *beta*-carotene. Molasses is an excellent carbon source for the production of carotene provided that the cultures are treated with *beta*-ionone. Without the addition of *beta*-ionone less carotene is produced than on the basal medium. The constituents in molasses responsible for enhancing the *beta*-ionone effect are not known. The addition of molasses ash or aconitic acid to the basal medium had little effect on carotene synthesis.

3. Nitrogen sources. The effect of adding 5 μl of *beta*-ionone to 3-day-old cultures grown in media containing various nitrogen sources was investigated. All of the media contained 0.19 g N/l. Representative data are presented in TABLE 2. Detailed data for two of the nitrogen sources are given in FIGS. 14 to 17.

It is evident that the magnitude of the *beta*-ionone effect depends upon the nitrogen source used in the medium. The *beta*-ionone effect was barely detectable when ammonium sulfate was used; this may be due

TABLE 2

THE EFFECT OF BETA-IONONE (5 μL/CULTURE) ON THE MAXIMUM YIELDS OF CAROTENE (AVERAGE OF TWO CULTURES) WHEN MIXED + AND − CULTURES WERE GROWN ON MEDIA CONTAINING DIFFERENT SOURCES OF NITROGEN. BETA-IONONE WAS ADDED ON THE THIRD DAY

Nitrogen source	Day	Control (μg/culture)	mg/g	Day	*beta*-Ionone (μg/culture)	mg/g	Per cent increase (μg/culture)
L-glutamic acid	11	93	1.50	9	360	6.25	287
Ammonium sulfate	15	7.2	0.18	15	8	0.22	11
L-asparagine	15	60	1.10	5	127	2.8	111
L-arginine	17	57	1.13	17	201	4.4	346
L-proline	9	64	0.93	13	288	4.56	346
DL-valine	14	39	0.77	14	166	4.3	325

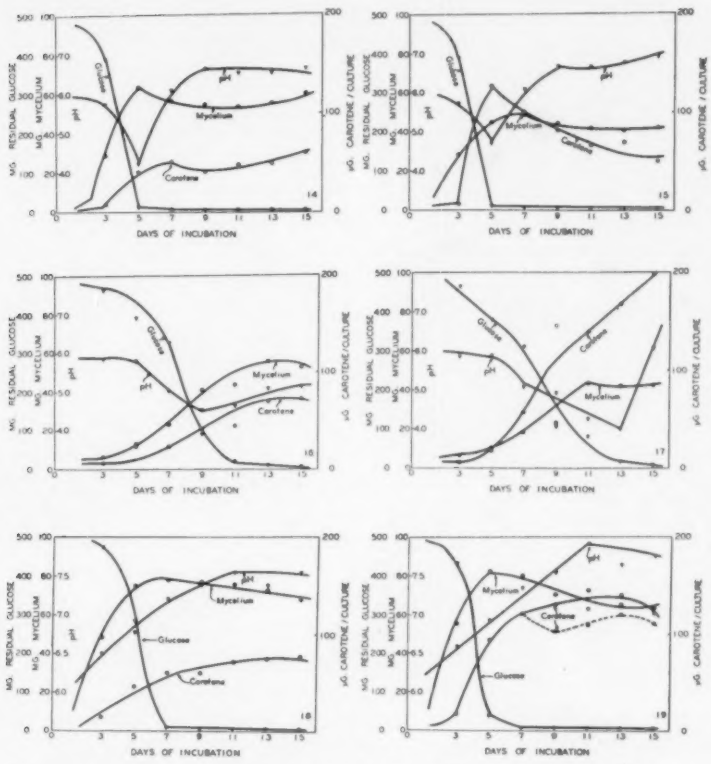
to the low pH levels of the culture medium (ca. 2.5). The addition of *beta*-ionone to 3-day-old cultures grown on the glucose-asparagine medium resulted in the maximum yield of carotene on the fifth day (FIG. 15). The maximum yield from cultures not treated with *beta*-ionone occurred on the fifteenth day (FIG. 14). The maximum yield of carotene from the cultures grown on the glucose-arginine medium and treated with *beta*-ionone occurred on the fifteenth day or later (FIG. 17); the same time was required by the control cultures (FIG. 16). The utilization of glucose was rapid in the asparagine and slow in the arginine medium. It is doubtful that the slow utilization of glucose in the arginine medium is the factor responsible for increased carotenogenesis since glucose utilization was complete in the glutamic acid medium in 7 days.

Environmental factors. The effects of temperature, light, and initial pH values of the medium were studied. Mixed + and − cultures were grown on the basal glucose-glutamic acid medium.

1. Temperature. Three temperatures were used. The maximum weights of mycelium were 82 mg (9 days), 78 mg (5 days) and 65 mg (7 days) when the temperature of incubation was 20, 25 and 28° C, respectively. The maximum yields of carotene at the three temperatures were 72 μg (20° C, 13 days), 85 μg (25° C, 17 days) and 93 μg (28° C, 11 days).

2. Light. In general, light has been reported to increase carotene synthesis by fungi. The basal medium and incubation at 25° C were used in these experiments. One set of cultures received some light (ca. 10 f.c. or less) 12 hours daily; the second set of cultures was kept in total darkness except when cultures were removed for harvest. A set of cultures kept in darkness for 7 days was transferred to continuous light (950 f.c.). The results are shown in FIGS. 18 and 19. Significantly more carotene accumulated in cultures kept in darkness (140 μg, 13 days) than in those exposed to alternate light and darkness (78 μg, 15 days). It is not known whether light catalyzed the destruction or inhibited the synthesis of carotene. *Choanephora cucurbitarum* appears to be unlike other fungi studied in that more carotene accumulates in cultures kept in darkness than in those exposed to some light.

3. Initial pH values. The failure of *Choanephora cucurbitarum* to synthesize more than a trace of carotene on the glucose-ammonium sulfate medium was attributed to the low pH values that developed. The effect of initial pH values on carotenogenesis was investigated using the basal medium. The initial pH values ranged from 3.2 to 7.8. Initial pH values of 5.6 or lower reduced the yield of carotene. The expected



FIGS. 14–19. FIG. 14 (upper left). Control, glucose-asparagine medium. FIG. 15 (upper right). Effects of adding 5 μl of *beta*-ionone to 3-day-old cultures grown on glucose-asparagine medium. FIG. 16 (center left). Control, glucose-arginine medium. FIG. 17 (center right). Effects of adding 5 μl of *beta*-ionone to 3-day-old cultures grown on glucose-arginine medium. FIG. 18 (lower left). Control cultures received 12 hr. light (ca. 10 f.c.) and 12 hr. darkness daily. FIG. 19 (lower right). Cultures kept in darkness (solid line); cultures moved to continuous light (dashed line). Mixed + and − cultures were used in all experiments.

yields of carotene were obtained when the initial pH values were between 6 and 7.

DISCUSSION

A number of pathways for the biosynthesis of carotenoids by fungi have been proposed (Goodwin, **1958**; Grob, **1956, 1958**). It is not known whether all fungi that produce carotenoids utilize the same pre-

cursors and pathways. If so, it must be said that the nutritional and environmental factors that favor carotenogenesis in different species differ. Thus, light depresses the yield of carotene by *Choanephora cucurbitarum,* and increases carotenogenesis in many other species including *Phycomyces blakesleeanus* (Garton et al., **1951**; Chichester et al., **1954**; Lilly et al., **1957**), and *Penicillium sclerotiorum* (Mase et al., **1957**). Zalokar (**1954**) found light essential for the production of carotenoids by *Neurospora crassa.* In this work it was found that pH values near 7 favored carotenogenesis by *C. cucurbitarum,* while low pH values (2.5 to 3.0) favored the production of carotene by *P. blakesleeanus* (Goodwin and Wilmer, **1952**; Friend et al., **1955**; Lilly et al., **1960**). Carotene production by *C. cucurbitarum* and other species of the Choanephoraceae (Barnett et al., **1956**; Hesseltine and Anderson, **1957**) is greatly increased when the two sexes are cultured together. Mixed + and − cultures of *P. blakesleeanus* (Lilly et al., **1957**) produced but little more carotene than the best single isolate. Mixed + and − cultures of *Mucor hiemalis* produced less carotene on a glucose-yeast extract medium, and produced more carotene on a glucose-asparagine medium, than the + and − isolates singly (Anderson, **1959**). A medium may be satisfactory for growth of *C. cucurbitarum* but unsatisfactory for carotene production. Similar results have been reported for *P. blakesleeanus* (Schopfer and Grob, **1952**) and *Blakeslea trispora* (Ciegler et al., **1959**). It appears that the conditions requisite for high yields of carotene are quite specific and that they differ in different fungi.

Like many other fungi, *Choanephora cucurbitarum* grows poorly on a medium containing acetate as the sole source of carbon. The rate at which acetate was utilized from a glucose-glutamic acid medium appeared to be slower than the utilization of acetate by *Phycomyces blakesleeanus* from a glucose-ammonium sulfate medium (Lilly et al., **1958, 1960**). The high pH value of the glucose-glutamic acid medium may have been responsible for the slower utilization of acetate by *C. cucurbitarum.*

Lipid synthesis in *Choanephora cucurbitarum* increases as the glucose concentration is increased. *C. cucurbitarum* produces more lipid material than *Phycomyces blakesleeanus.* Garton et al. (**1951**) reported *P. blakesleeanus* mycelium to contain about 19 per cent of lipids when the medium contained 100 g/l of glucose. The mycelium of *C. cucurbitarum* contained about 50 per cent of lipids when grown in a medium containing 100 g/l of glucose. Is carotenogenesis related in any way to lipid synthesis or utilization? In the absence of glucose the lipid content of *P. blakesleeanus* mycelium decreased (Goodwin and Lijinsky, **1951**). The carotene content of the mycelium decreased also. These authors

transferred mycelium of *P. blakesleeanus* to media which contained 1 per cent of L-valine, L-leucine, or L-asparagine. The weights of the mycelium and the lipid content decreased (3 to 6 days) while the carotene content increased. Whether amino acid or lipid carbon or both were used for carotene synthesis is not known. *Choanephora cucurbitarum* continued to synthesize carotene for a time after the glucose was utilized. If *C. cucurbitarum* oxidizes fatty acids by *beta*-oxidation, such a process would provide acetate for the synthesis of carotene. The addition of corn oil (Mazola) to agitated cultures of *C. cucurbitarum* inhibited with diphenylamine resulted in a 5-fold increase in carotene production (Anderson, **1959**).

MacKinney et al. (**1953**) observed that *beta*-ionone depressed the growth of *Phycomyces blakesleeanus* slightly and reduced the rate at which glucose was utilized. Ciegler et al. (**1959a**) found *beta*-ionone to reduce growth of *Blakeslea trispora* slightly. An extensive series of experiments with *Choanephora cucurbitarum* showed that the effects of *beta*-ionone depended on the amount added and the time of addition. The magnitude of the *beta*-ionone effect was also shown to depend on the medium used. *beta*-Ionone appears to be a toxic substance which inhibits growth, glucose utilization, zygospore formation, lipid synthesis, and aerobic respiration and increases the yield of carotene. In view of the inhibition of growth, respiration, lipid synthesis, and glucose utilization it may be supposed that these restrictions leave more of certain intermediates available for carotene synthesis. It is not known how *beta*-ionone acts, but it may be supposed that the action of, and perhaps the synthesis of, certain enzymes are decreased.

It is of interest that the nature of the medium affects the *beta*-ionone effect. Little or no effect was observed with cultures grown on the glucose-ammonium sulfate medium (pH 2.6 at the time of addition). Lilly et al. (**1960**) working with *Phycomyces blakesleeanus* found that *beta*-ionone stimulated carotenogenesis more when the pH was near 5 than when it was 2.5 to 2.7. It is to be noted also that the carotene production of cultures grown on various media in the absence of *beta*-ionone does not indicate how effective *beta*-ionone will be. Thus, less carotene was produced on the molasses-glutamic acid medium than the glucose-glutamic acid medium when the cultures were not treated with *beta*-ionone. Yet, *beta*-ionone-treated cultures grown on the molasses-glutamic acid medium produced more carotene than treated cultures grown on the glucose-glutamic acid medium. Treating cultures grown on the glucose-asparagine medium resulted in a rapid increase in carotene content which ceased after 2 days. *beta*-Ionone treated cultures

grown on the glucose-arginine medium continued to produce carotene until the fifteenth day of incubation.

To obtain maximum yields of carotene it is important to add *beta*-ionone after considerable mycelium has formed, but before growth is complete. Ciegler et al. (**1959a**) also note that the time of *beta*-ionone addition is critical for *Blakeslea trispora*. From the carotene production data it is presumed that the substances produced by the + and − mycelia which stimulate carotenogenesis in the mycelium of the opposite sex are formed abundantly at this time. Experiments in which the sexes were cultured separately and where the media were interchanged did not lead to a perceptible increase in the synthesis of carotene. Presumably, mutual and continued interchange of these substances is necessary for the production of the compounds which increase carotenogenesis.

SUMMARY

Mixed + and − cultures produced 3 to 5 times as much carotene as either isolate produced when grown alone. Increasing the glucose concentration from 20 to 80 g/l more than doubled the carotene yield from mixed + and − cultures; this increase in glucose concentration resulted in an insignificant increase in carotene production when the + and − isolates were grown separately. Hexoses (glucose, mannose, and galactose) were the best carbon sources for carotene production by mixed + and − cultures. Glutamic acid was the best nitrogen source. The lipid content of the mycelium increased as the glucose content of the medium increased; the lipid content was approximately 50 per cent when the medium contained 100 g/l of glucose. Carotene synthesis continued for several days after the glucose was utilized; the lipid content decreased after the glucose was exhausted. The addition of potassium acetate to 3-day-old cultures grown on the basal medium increased carotene production somewhat. Experiments with acetate-1-C^{14} showed that acetate was utilized. *beta*-Ionone stimulated carotenogenesis markedly when it was added to half-grown + and − cultures; it was relatively ineffective when added to separate cultures of either isolate. In addition, *beta*-ionone inhibited growth, decreased the rate of glucose utilization and lipid synthesis, reduced the rate of respiration, and delayed zygospore formation and maturation. Yields of carotene slightly in excess of 7 mg/g dry mycelium were obtained from 4-day-old cultures grown on a molasses-glutamic acid medium and which were treated with 5 μl of *beta*-ionone per culture on the second day of incubation.

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LITERATURE CITED

Anderson, C. D. 1959. The role of carotenoid pigments in fungi. Dissertation. West Virginia University, Morgantown. 152 pp.

Anderson, R. F., Margie Arnold, G. E. N. Nelson, and A. Ciegler. 1958. Microbiological production of *beta*-carotene in shaken flasks. Agr. Food Chem. **6**: 543–545.

Barnett, H. L., and V. G. Lilly. 1950. Influence of nutritional and environmental factors upon asexual reproduction of *Choanephora cucurbitarum* in culture. Phytopathology **40**: 80–89.

——, **and** ——. 1955. The effects of humidity, temperature, and carbon dioxide on sporulation of *Choanephora cucurbitarum*. Mycologia **47**: 20–29.

——, **and** ——. 1956. Factors affecting the production of zygospores by *Choanephora cucurbitarum*. Mycologia **48**: 617–627.

——, ——, **and R. F. Krause.** 1956. Increased production of carotene by mixed + and − cultures of *Choanephora cucurbitarum*. Science **123**: 141.

Chichester, C. O., Patricia S. Wong, and G. MacKinney. 1954. On the biosynthesis of carotenoids. Plant Physiol. **29**: 238–241.

Ciegler, A., Margie Arnold, and R. F. Anderson. 1959. Microbiological production of carotenoids. 4. Effect of various grains on the production of *beta*-carotene by mated strains of *Blakeslea trispora*. App. Microbiol. **7**: 94–98.

——, ——, **and** ——. 1959a. Microbiological production of carotenoids. 5. Effects of lipids and related substances on the production of *beta*-carotene. App. Microbiol. **7**: 98–101.

Engel, B. G., J. Würsch, and M. Zimmerman. 1953. Über den Einfluss von *beta*-Jonon auf die Bildung von *beta*-Carotin durch *Phycomyces blakesleeanus*. Hel. Chim. Acta **36**: 1771–1776.

Friend, J., T. W. Goodwin, and L. A. Griffiths. 1955. Studies in carotenogenesis. 15. The role of carboxylic acids in the biosynthesis of *beta*-carotene by *Phycomyces blakesleeanus*. Biochem. Jour. **60**: 649–655.

Garton, G. A., T. W. Goodwin, and W. Lijinsky. 1951. Studies in carotenogenesis. 1. General conditions governing *beta*-carotene synthesis by the fungus *Phycomyces blakesleeanus* Burgeff. Biochem. Jour. **48**: 154–163.

Goodwin, T. W. 1952. Fungal carotenoids. Botan. Rev. **18**: 291–316.

——. 1954. Carotenoids, their comparative biochemistry. Chemical Publishing Co. New York. 356 pp.

——. 1955. Carotenoids. Ann. Rev. Biochem. **24**: 497–522.

——. 1958. Carotenoids. Encyclopedia of Plant Physiology **10**: 186–222. Springer-Verlag. Berlin-Göttingen-Heidelberg.

——, **and W. Lijinsky.** 1951. Studies in carotenogenesis. 2. Carotene production by *Phycomyces blakesleeanus:* the effect of different amino-acids when used in media containing low concentrations of glucose. Biochem. Jour. **50**: 268–273.

——, **and J. S. Willmer.** 1952. Studies in carotenogenesis. 4. Nitrogen metabolism and carotene synthesis in *Phycomyces blakesleeanus*. Biochem. Jour. **51**: 213–217.

Grob, E. C. 1956. Über die Biosynthese der Carotinoide bei einigen Mikroorganismen. Chemia **10**: 73–80.

——. 1958. Über die Bildung der Carotinoide bei *Neurospora crassa*. Chemia **12**: 86–88.

——, **and R. Bütler.** 1954. Über die Biosynthese des *beta*-Carotins bei *Mucor hiemalis*. Die Beteiligung der Essigsäure am Aufbau des Carotinmoleküls, untersucht mit Hilfe C^{14}-markierter Essigsäure. Experientia **10**: 73–80.

Hesseltine, C. W., and R. F. Anderson. 1957. Microbiological production of carotenoids. 1. Zygospores and carotene produced by intraspecific and interspecific crosses of Choanephoraceae in liquid media. Mycologia **49**: 449–452.

Keller, A. G., Jr. 1951. University of Pennsylvania manual of biochemical laboratories of the graduate hospital, University of Pennsylvania, Philadelphia. 173 pp.

Lilly, V. G., H. L. Barnett, and R. F. Krause. 1957. The effects of light and temperature on the production of carotenoid pigments by + and − sexes of *Phycomyces blakesleeanus*. Proc. W. Va. Acad. Sci. **29**: 29–31.

——, ——, **and** ——. 1960. The production of carotene by *Phycomyces blakesleeanus*. W. Va. Agric. Expt. Sta. Bull. 441 T.

——, ——, ——, **and F. J. Lotspeich.** 1958. A method of obtaining pure radioactive *beta*-carotene using *Phycomyces blakesleeanus*. Mycologia **50**: 862–873.

MacKinney, G., T. Nakayama, C. D. Buss, and C. O. Chichester. 1952. Carotenoid production in *Phycomyces*. Jour. Amer. Chem. Soc. **74**: 3456–3457.

——, ——, **C. O. Chichester, and C. D. Buss.** 1953. Biosynthesis of carotene in *Phycomyces*. Jour. Amer. Chem. Soc. **75**: 236–238.

Mase, Y., W. J. Rabourn, and F. W. Quackenbush. 1957. Carotene production by *Penicillium sclerotiorum*. Arch. Biochem. and Biophys. **68**: 150–156.

Schopfer, W. H., and E. C. Grob. 1952. Sur la biosynthèse du *beta*-carotène par *Phycomyces blakesleeanus* cultivé sur un millieu contenant de l'acetate de sodium comme unique source de carbone. Experientia **8**: 140.

Wolf, F. A. 1917. A squash disease caused by *Choanephora cucurbitarum*. Jour. Agric. Res. **8**: 319–328.

Zalokar, M. 1954. Studies on biosynthesis of carotenoids in *Neurospora crassa*. Arch. Biochem. and Biophys. **50**: 71–80.

THE MORPHOLOGY AND A CHEMICAL ANALYSIS OF THE TELIOSPORE OF THE DWARF BUNT FUNGUS, TILLETIA CONTRAVERSA [1]

S. O. GRAHAM

(WITH 3 FIGURES)

INTRODUCTION

In a recent study on the responses of dwarf bunt teliospore walls to reagents and dyes (**7**), it was noted that Nile blue A stained a "mesospore" layer red. As early as 1898 van Wisselingh (**21**) described three parts to the teliospore wall of *Tilletia rauwenhoffiii* Fisch. v. Waldh. using his qualitative mycosin (chitosan) reaction. Meiners (**13**), employing some of van Wisselingh's procedures on several smut species including dwarf bunt (*Tilletia contraversa* Kühn), distinguished a well-defined inner layer (endospore) which proved to be highly chitinous and an outer reticulate layer (exospore) which was relatively free of chitin. He was unable to demonstrate a third distinct layer exclusive of any sheath component.

In earlier studies (**6**, **7**), differential solubilities and histochemical tests on certain parts of the teliospore wall indicated it feasible to define chemical and structural components at least in part. This endeavor was undertaken to separate the various wall layers of dwarf bunt teliospores and to determine their chemical compositions.

MATERIALS AND METHODS

The close relationships between the methods necessary to separate wall parts and those used to determine their chemical compositions were such that the two aspects were usually considered simultaneously. Histochemical procedures were usually performed on spore residues after treatments to determine what materials remained. Specific chemical tests were run on leachates and supernatant fractions to determine what materials they contained.

The chemical contexts of wall parts, based on materials identifiable in soluble fractions and residues, were determined in part by modifications

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of the procedures of Thomas (**18, 19, 20**). Selected reagents and procedures that were used to identify materials in fractions from specific treatments, or the particular histochemical methods that were employed on residues, were patterned after those in references as listed below.

Carbohydrates	
General	—Molisch's test (**10, 14**)
Cellulose	—Schweitzer's reagent (**10, 16**)
	—Iodine-Potassium iodide and sulfuric acid (**16**)
	—Cellulase (**15**)
Hexoses	—Tauber's reagent (**14**)
Pentoses	—Tauber's reagent (**14**)
	—Tollen's orcinol reaction (**10**)
Pectins	—Ruthenium red (**16, 9**)
	—Methylene blue (**9**)
	—Prussian blue reaction (**16**)
	—Safranin O (**9, 14**)
	—Pectinase digestion (**9, 16**)
	—Alcoholic potash digestion (**18, 19**)
	—Ammonium oxalate digestion (**18, 19**)
	—Phloroglucin-hydrochloric acid (**10**)
Hemicellulose	—Resorcin blue (**18, 19**)
	—Methylene blue (**9, 14**)
	—Hemicellulase digestion (**15**)
Reducing sugars	—Benedict's solution (**10**)
Sucrose	—Raybin's diazouracil test (**14**)
Proteins	
General	—Trichloroacetic acid (**10**)
	—Biuret reaction (**10**)
	—Xanthoproteic test (**10, 16**)
	—Millon's reaction (**10**)
Amino acids	—Ninhydrin reaction (**16**)
Melanins	—Ferric chloride precipitation (**10**)
	—Trichloroacetic acid (**10**)
	—Ninhydrin (**10**)
	—Argentaffin Test (Fontana's silver solution) (**5**)
	—Millon's reaction (**10**)
Lignins	—Iodine-Potassium iodide and sulfuric acid (**16**)
	—Phloroglucinol reaction (**16**)
	—Mäule reaction (**16**)
Chitin	—Zander's solution (**4**)
	—Chitosan reaction (**21, 16**)
Lipids	—Acrolein test for glycerol (**10**)
	—Molybdate test for phospholipids (**10**)
	—Salkowski's reaction for ergosterol (**10**)
	—Oil red O (**2**)
	—Sudan black B (**16, 2**)
	—Sudan IV (**16, 2**)
	—Nile blue A (**5, 16**)

A. *Determining Soluble Fractions and Tests on Spore Residues after Extractions*

1. *Procedure.*

Twenty-two grams of *Tilletia contraversa* teliospores free from peridium material were leached six hours in 100 ml distilled water over a magnestirrer at room temperature. The sample was centrifuged and the supernatant was collected and refrigerated. One hundred milliliters of distilled water was again added to the sample. It was allowed to leach overnight over a magnestirrer at room temperature, and the sample was again centrifuged to collect spores. The supernatant was combined with the previous leachate and labeled the water soluble fraction for later analysis. Free and loosely bound proteins, carbohydrates and related compounds were expected in this fraction. A small sample of spores was set aside for histochemical tests.

The residue was then refluxed for one hour in 75 ml of 0.5% ammonium oxalate solution over low heat to extract calcium. The sample was centrifuged to collect spores and the supernatant was collected. This procedure was repeated twice. The supernatants were combined as the ammonium oxalate supernatant No. 1. Pectic substances were expected in this fraction.

The spore residue was washed twice with warm distilled water to remove oxalate. The wash was discarded and a small sample of spores was set aside for histochemical tests.

The spore residue was then digested for 24 hours over a magnestirrer in 150 ml of cold ammoniacal copper hydroxide (Schweitzer's reagent). The sample was made acid with 5% acetic acid, centrifuged and the supernatant kept as Schweitzer Supernatant No. 1. Proteins and polysaccharides were expected in this fraction. The spore residue was washed twice in 5% acetic acid to remove any residual ammoniacal copper hydroxide and washed twice again in distilled water to remove the acetic acid. A sample of spores was set aside for histochemical tests.

The spore residue was refluxed for another hour over low heat in 0.5% ammonium oxalate and centrifuged. The supernatant was collected and stored as ammonium oxalate supernatant No. 2. Additional pectin was expected in this fraction. The residue was washed twice with warm distilled water to remove oxalate and a small sample of spores was set aside for histochemical tests.

The remaining spore residue was washed twice with 95% ethyl alcohol and refluxed overnight in 150 ml of 95% ethyl alcohol. The sample was centrifuged and the supernatant collected. The residue was evapo-

rated to dryness under vacuum and leached for 4 hours in ethyl ether by refluxing over a hot water bath. The sample was centrifuged and the supernatant collected. It was combined with the alcohol supernatant and kept as the alcohol-ether soluble fraction. Lipid materials were expected in this fraction. A small sample of spores was set aside for histochemical tests.

The spore residue was washed twice with cold ether, dried under vacuum to remove the remaining solvent, and refluxed for 24 hours in 200 ml of 4% alcoholic potash (4 grams potassium hydroxide per 100 ml 95% ethyl alcohol). The sample was centrifuged to collect spores and the supernatant was kept as the alcoholic potash-soluble fraction. The residue was washed twice with 95% ethyl alcohol and the supernatants obtained were combined with the alcoholic potash fraction. Additional pectic materials and hemicelluloses were expected in this fraction. A small sample of the spore residue was set aside for histochemical tests.

2. *Tests and Results on Supernatants and Spore Residues.*

a. *The Water-Soluble Fraction.*

This fraction was tested primarily for carbohydrates and proteins. The solution was discolored yellow by a pigment which was non-ether-soluble. It was easily removed by Darco G-60 decolorizing charcoal. No attempts were made to determine the pigment in this fraction.

Tests for carbohydrates on the decolorized supernatant were positive. The Molisch's test was very strong. The Tauber's test for hexoses was positive. Tollen's orcinol reaction for pentoses was negative. The Benedict's test for reducing sugars was positive, and the Raybin diazo-uracil test for sucrose (or raffinose, gentianose, and stachyose) was negative.

A portion of the fraction was reduced to one-third by vacuum distillation and stained pink with a 1:1000 solution of Ruthenium red dye. Two volumes of 95% ethyl alcohol were added. If the solution contained dissolved pectic material, any precipitate produced would be red. No red or pink precipitate was obtained.

Tests for proteins were negative or inconclusive, but amino acids were present. The Biuret reaction on the decolorized fraction was inconclusive. Trichloroacetic acid produced a precipitate that gave a positive Molisch's reaction after some time, but the Biuret reaction on it was still slow and inconclusive. A positive ninhydrin reaction was obtained on the original decolorized fraction, but was negative on neutralized filtrate after trichloroacetic acid. The washed and neutralized precipitate produced by trichloroacetic acid was negative to the ninhydrin reaction, as well. Presumably trichloroacetic acid interfered

with the reaction. The xanthoproteic test for tyrosine or tryptophane was negative. A positive Millon's reaction was obtained on the non-decolorized fraction. This was presumably owing to the yellow pigment which could have been phenolic in nature. The reaction was negative on the decolorized filtrate.

b. *Tests on the Spore Residue of the Water-Soluble Fraction.*

Spores were mounted and observed in a 1:10,000 concentration of freshly prepared aqueous Ruthenium red dye in 50% alcohol. The sheath, the reticulum, and numerous protoplasts fixed the dye. The structures retained the stain upon heating, although the sheath was considerably faded. Thus, undissolved pectic materials were in these structures. Although the stain was relatively light at this concentration (standard concentration is 1:5000), it was necessary, as the standard concentration was very astringent to sheath materials and obscured structures.

Owing to the presence of pectins, spores were easily mordanted with iron to obtain a Prussian blue reaction. This reaction was positive in the same areas as those found to fix Ruthenium red dye. In addition, however, a lighter staining area was defined just outside the protoplast in the endospore wall, indicating the gross endospore structure was not homogeneous.

To note if the pigment of the reticulate layer was perhaps a melanin and hence phenolic in nature, or if phenolic constituents of a protein nature (tyrosine, tryptophane, etc.) were present, the argentaffin test was applied. Silver was reduced as a black deposit in the reticulate layer. It was reduced as a silvery-grey precipitate in the protoplast. However, the sheath and endospore layers appeared non-responsive. Thus, phenolic compounds were present in the reticulum and the protoplast, although they were probably of different natures.

Spores were mounted in 0.25% ninhydrin reagent in pH 7 buffer containing a trace of pyridine. The mount was heated over a steam bath for five minutes. A positive reaction was obtained for alpha amino acids in the reticulum and the protoplast, indicating the presence of proteins there. There was no color produced in the sheath or in the endospore.

The Millon's reaction for mono-hydroxy benzene derivatives, including melanin pigments and proteins containing tyrosine, was applied histochemically. It was positive in the reticulum. The test was inconclusive for the protoplast. For clarification, the xanthoproteic test for proteins containing tyrosine or tryptophane was applied histochemically. No yellow or orange color was produced in the reticulum, but an orange-pink color was produced in the protoplast. Thus, the Millon's reaction

was considered positive for phenolic pigment rather than for any protein containing tyrosine or tryptophane.

Lipophilic stains varied, depending upon the dye employed. Nile blue A stained the reticulum green-blue but stained a fine band of material pink between the reticulum and the endospore, indicating a neutral lipoid layer (e.g., triglycerides, sterols, waxes or carotenoids) free of acidic lipoids at that site. Oil globules within the spore also stained pink, and numerous free-floating oil globules in the mounts stained pink as well. Whether these globules were independent lipoids or whether they were escaped globules from crushed spores could not be ascertained.

Oil red O faintly stained sheath and reticular elements pink, but it was not deposited as a definable band between the reticulum and the endospore. It stained internal oil globules only if the spore was crushed. Penetration is apparently limiting in the use of this dye. It was observed that free-floating oil globules had a much greater affinity for the dye than did the oil globules expressed from spore protoplasts, suggesting a different nature of the two sources of lipoids.

Sudan black B stained sheath and reticular elements a faint light-grey. It stained a fine band of material between the reticulum and endospore a blue-black. Free-floating oil globules were stained a dense blue-black. Internal oil globules of the spores did not take up the dye unless spores were crushed. In this case they stained as dense as free-floating globules.

Sudan IV in Shear's mounting fluid (**1**) stained free-floating oil globules a bright orange, but did not appear to be fixed in any spore part except in the oil globules of crushed spores. There was no apparent difference in dye uptake between the two sources of lipoids. Thus, any phospholipid present in the spore wall would not be lecithin, since this material, pure or as a mixture, fixes Sudan stains but not Nile blue A (**8**).

Resorcin blue dye, considered specific for callose (a hemicellulose), was not fixed in spores. It was fixed lightly in sterile cells, both in the walls and protoplasts. Methylene blue was readily fixed in all spore parts. However, upon adding 2% hydrochloric acid to the edge of the cover slip, the stain disappeared from the sheath and endospore, indicating only polysaccharides there, but nitrogenous materials in the reticulum and protoplast (**9**).

Tests for lignin using the Mäule reaction were negative, and the phloroglucinol test for the material was negative. After oxidizing and acidifying in procedures of the Mäule reaction, Safranin dye was added to a mount before administering the sodium bicarbonate. The alkaline conditions altered the color of the dye variably in different wall parts.

Two layers to the endospore were differentiated.[2] The outer portion stained red while the inner portion stained yellow-orange, indicating localization of a reducing carbohydrate proximal to the protoplast (**14**). The sheath was stained yellow, indicative of reducing carbohydrate there. The reticulum, however, was stained pink at the periphery, fading to colorless at the base. The protoplast retained the original opaque red. The various structures were considerably swelled by the oxidation of the reaction.

The chitosan reaction was performed on a sample of spores. Chitin was present in the protoplast and in the endospore. There was none of the material defined in the reticular layer. Spores were void of any sheath and the reticular layer sloughed-off very easily when the coverslip was agitated (FIG. 1C). It was found to be truly net-like (FIG. 1D).

The endospore was differentiated into two well-defined layers (similar to FIG. 1A before agitating the coverslip), and was separable upon agitating the coverslip (FIG. 1C). The outer layer was more violet than the maroon-colored inner layer. The disorganized protoplast was an opaque maroon.

A portion of the sample that had been digested for three hours in the autoclave in 23 M potassium hydroxide for the chitosan reaction was washed in 50% alcohol to remove most of the alkali and further hardened in 70% alcohol. It was then placed in 5% sulphuric acid to neutralize and transferred to pH 7 buffer. Some of the spores were mounted in Resorcin blue (1:1000 lacmoid in 50% alcohol) and placed over 50% alcohol in a covered container held at 25° C for 24 hours. Other spores were mounted in Ruthenium red (1:5000 in 50% alcohol). Spores failed to fix either stain in any part.

Others of the neutralized spores were placed on a mount and observed while the oxidizing agent sodium hypochlorite (commercial Clorox) was added to the edge of the coverslip. The reticulum expanded away from the endospore rapidly, obtaining two to three diameters its original dimensions. In time the reticulum dissolved. The endospore became greatly enlarged too, but expanded to a lesser degree than did the reticulum. Ultimately the enlarged endospores ruptured to exude the contents. The exuded material was water insoluble, but it was immediately dissolved by Shear's mounting fluid (**1**), when the medium was added to the edge of the coverslip.

After lysis the residual endospore material shrank considerably and the structure was observed to be much thinner. It failed to fix those

[2] These layers should not be confused with the spherical aberrations often presented by spores in mounts.

rated to dryness under vacuum and leached for 4 hours in ethyl ether by refluxing over a hot water bath. The sample was centrifuged and the supernatant collected. It was combined with the alcohol supernatant and kept as the alcohol-ether soluble fraction. Lipid materials were expected in this fraction. A small sample of spores was set aside for histochemical tests.

The spore residue was washed twice with cold ether, dried under vacuum to remove the remaining solvent, and refluxed for 24 hours in 200 ml of 4% alcoholic potash (4 grams potassium hydroxide per 100 ml 95% ethyl alcohol). The sample was centrifuged to collect spores and the supernatant was kept as the alcoholic potash-soluble fraction. The residue was washed twice with 95% ethyl alcohol and the supernatants obtained were combined with the alcoholic potash fraction. Additional pectic materials and hemicelluloses were expected in this fraction. A small sample of the spore residue was set aside for histochemical tests.

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a. *The Water-Soluble Fraction.*

This fraction was tested primarily for carbohydrates and proteins. The solution was discolored yellow by a pigment which was non-ether-soluble. It was easily removed by Darco G-60 decolorizing charcoal. No attempts were made to determine the pigment in this fraction.

Tests for carbohydrates on the decolorized supernatant were positive. The Molisch's test was very strong. The Tauber's test for hexoses was positive. Tollen's orcinol reaction for pentoses was negative. The Benedict's test for reducing sugars was positive, and the Raybin diazo-uracil test for sucrose (or raffinose, gentianose, and stachyose) was negative.

A portion of the fraction was reduced to one-third by vacuum distillation and stained pink with a 1:1000 solution of Ruthenium red dye. Two volumes of 95% ethyl alcohol were added. If the solution contained dissolved pectic material, any precipitate produced would be red. No red or pink precipitate was obtained.

Tests for proteins were negative or inconclusive, but amino acids were present. The Biuret reaction on the decolorized fraction was inconclusive. Trichloroacetic acid produced a precipitate that gave a positive Molisch's reaction after some time, but the Biuret reaction on it was still slow and inconclusive. A positive ninhydrin reaction was obtained on the original decolorized fraction, but was negative on neutralized filtrate after trichloroacetic acid. The washed and neutralized precipitate produced by trichloroacetic acid was negative to the ninhydrin reaction, as well. Presumably trichloroacetic acid interfered

with the reaction. The xanthoproteic test for tyrosine or tryptophane was negative. A positive Millon's reaction was obtained on the non-decolorized fraction. This was presumably owing to the yellow pigment which could have been phenolic in nature. The reaction was negative on the decolorized filtrate.

b. *Tests on the Spore Residue of the Water-Soluble Fraction.*

Spores were mounted and observed in a 1:10,000 concentration of freshly prepared aqueous Ruthenium red dye in 50% alcohol. The sheath, the reticulum, and numerous protoplasts fixed the dye. The structures retained the stain upon heating, although the sheath was considerably faded. Thus, undissolved pectic materials were in these structures. Although the stain was relatively light at this concentration (standard concentration is 1:5000), it was necessary, as the standard concentration was very astringent to sheath materials and obscured structures.

Owing to the presence of pectins, spores were easily mordanted with iron to obtain a Prussian blue reaction. This reaction was positive in the same areas as those found to fix Ruthenium red dye. In addition, however, a lighter staining area was defined just outside the protoplast in the endospore wall, indicating the gross endospore structure was not homogeneous.

To note if the pigment of the reticulate layer was perhaps a melanin and hence phenolic in nature, or if phenolic constituents of a protein nature (tyrosine, tryptophane, etc.) were present, the argentaffin test was applied. Silver was reduced as a black deposit in the reticulate layer. It was reduced as a silvery-grey precipitate in the protoplast. However, the sheath and endospore layers appeared non-responsive. Thus, phenolic compounds were present in the reticulum and the protoplast, although they were probably of different natures.

Spores were mounted in 0.25% ninhydrin reagent in pH 7 buffer containing a trace of pyridine. The mount was heated over a steam bath for five minutes. A positive reaction was obtained for alpha amino acids in the reticulum and the protoplast, indicating the presence of proteins there. There was no color produced in the sheath or in the endospore.

The Millon's reaction for mono-hydroxy benzene derivatives, including melanin pigments and proteins containing tyrosine, was applied histochemically. It was positive in the reticulum. The test was inconclusive for the protoplast. For clarification, the xanthoproteic test for proteins containing tyrosine or tryptophane was applied histochemically. No yellow or orange color was produced in the reticulum, but an orange-pink color was produced in the protoplast. Thus, the Millon's reaction

was considered positive for phenolic pigment rather than for any protein containing tyrosine or tryptophane.

Lipophilic stains varied, depending upon the dye employed. Nile blue A stained the reticulum green-blue but stained a fine band of material pink between the reticulum and the endospore, indicating a neutral lipoid layer (e.g., triglycerides, sterols, waxes or carotenoids) free of acidic lipoids at that site. Oil globules within the spore also stained pink, and numerous free-floating oil globules in the mounts stained pink as well. Whether these globules were independent lipoids or whether they were escaped globules from crushed spores could not be ascertained.

Oil red O faintly stained sheath and reticular elements pink, but it was not deposited as a definable band between the reticulum and the endospore. It stained internal oil globules only if the spore was crushed. Penetration is apparently limiting in the use of this dye. It was observed that free-floating oil globules had a much greater affinity for the dye than did the oil globules expressed from spore protoplasts, suggesting a different nature of the two sources of lipoids.

Sudan black B stained sheath and reticular elements a faint light-grey. It stained a fine band of material between the reticulum and endospore a blue-black. Free-floating oil globules were stained a dense blue-black. Internal oil globules of the spores did not take up the dye unless spores were crushed. In this case they stained as dense as free-floating globules.

Sudan IV in Shear's mounting fluid (**1**) stained free-floating oil globules a bright orange, but did not appear to be fixed in any spore part except in the oil globules of crushed spores. There was no apparent difference in dye uptake between the two sources of lipoids. Thus, any phospholipid present in the spore wall would not be lecithin, since this material, pure or as a mixture, fixes Sudan stains but not Nile blue A (**8**).

Resorcin blue dye, considered specific for callose (a hemicellulose), was not fixed in spores. It was fixed lightly in sterile cells, both in the walls and protoplasts. Methylene blue was readily fixed in all spore parts. However, upon adding 2% hydrochloric acid to the edge of the cover slip, the stain disappeared from the sheath and endospore, indicating only polysaccharides there, but nitrogenous materials in the reticulum and protoplast (**9**).

Tests for lignin using the Mäule reaction were negative, and the phloroglucinol test for the material was negative. After oxidizing and acidifying in procedures of the Mäule reaction, Safranin dye was added to a mount before administering the sodium bicarbonate. The alkaline conditions altered the color of the dye variably in different wall parts.

Two layers to the endospore were differentiated.[2] The outer portion stained red while the inner portion stained yellow-orange, indicating localization of a reducing carbohydrate proximal to the protoplast (**14**). The sheath was stained yellow, indicative of reducing carbohydrate there. The reticulum, however, was stained pink at the periphery, fading to colorless at the base. The protoplast retained the original opaque red. The various structures were considerably swelled by the oxidation of the reaction.

The chitosan reaction was performed on a sample of spores. Chitin was present in the protoplast and in the endospore. There was none of the material defined in the reticular layer. Spores were void of any sheath and the reticular layer sloughed-off very easily when the coverslip was agitated (FIG. 1C). It was found to be truly net-like (FIG. 1D).

The endospore was differentiated into two well-defined layers (similar to FIG. 1A before agitating the coverslip), and was separable upon agitating the coverslip (FIG. 1C). The outer layer was more violet than the maroon-colored inner layer. The disorganized protoplast was an opaque maroon.

A portion of the sample that had been digested for three hours in the autoclave in 23 M potassium hydroxide for the chitosan reaction was washed in 50% alcohol to remove most of the alkali and further hardened in 70% alcohol. It was then placed in 5% sulphuric acid to neutralize and transferred to pH 7 buffer. Some of the spores were mounted in Resorcin blue (1:1000 lacmoid in 50% alcohol) and placed over 50% alcohol in a covered container held at 25° C for 24 hours. Other spores were mounted in Ruthenium red (1:5000 in 50% alcohol). Spores failed to fix either stain in any part.

Others of the neutralized spores were placed on a mount and observed while the oxidizing agent sodium hypochlorite (commercial Clorox) was added to the edge of the coverslip. The reticulum expanded away from the endospore rapidly, obtaining two to three diameters its original dimensions. In time the reticulum dissolved. The endospore became greatly enlarged too, but expanded to a lesser degree than did the reticulum. Ultimately the enlarged endospores ruptured to exude the contents. The exuded material was water insoluble, but it was immediately dissolved by Shear's mounting fluid (**1**), when the medium was added to the edge of the coverslip.

After lysis the residual endospore material shrank considerably and the structure was observed to be much thinner. It failed to fix those

[2] These layers should not be confused with the spherical aberrations often presented by spores in mounts.

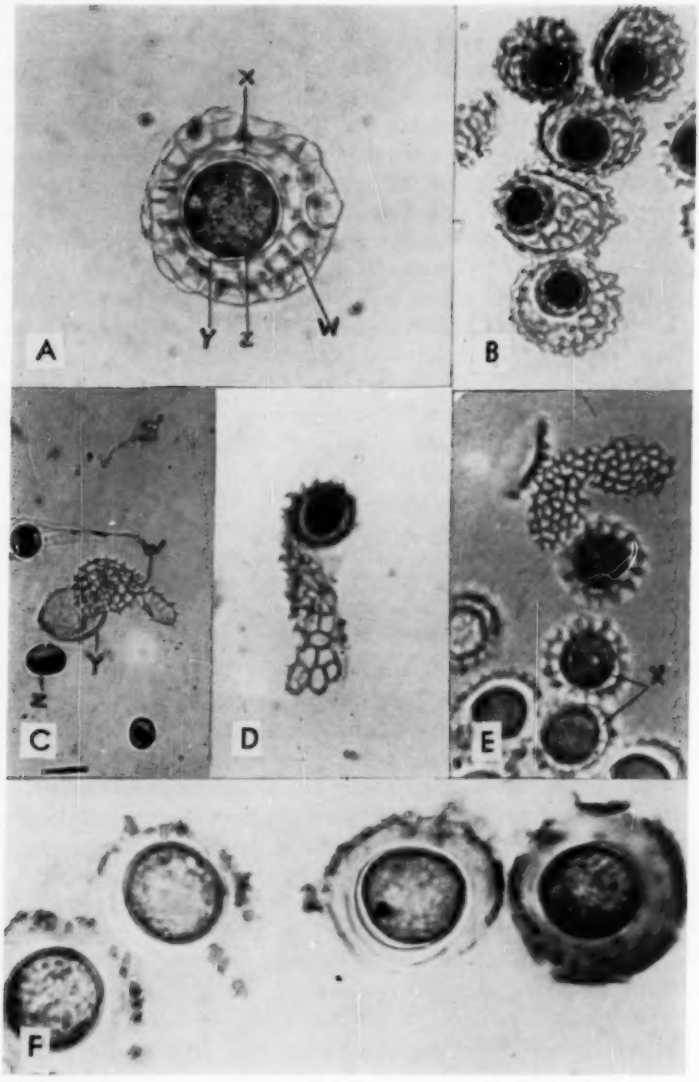

FIG. 1. Layers of the dwarf bunt teliospore wall. A and E. Unfixed spores oxidized with NaClO followed by KOH and stained with Safranin O (w-expanded reticulum, x-viscous globule between the reticulum and endospore, y-outer endospore layer, z-inner endospore layer). B. Spores treated similar to A after previous leaching in ether, showing absence of viscous component. C and D. Separated wall parts of unfixed spores after the chitosan reaction. F. Spores in the process of dissolution after being mounted first in KOH and then oxidized with NaClO.

dyes that the endospore normally does fix. The only dye that the structure would fix well, of those tried, was Rose bengal.

c. *The Ammonium Oxalate Supernatant.*

This supernatant was expected to contain pectic materials. It was strongly stained by a brown, ether-insoluble pigment which was incompletely removed by decolorizing charcoal (Darco G-60). The essentially decolorized solution reacted negatively under the ninhydrin reaction [3] and the biuret reaction. The Molisch's test for carbohydrates and the Benedict's test for reducing sugars were positive; Tauber's test for hexose, pentose, or uronic acids was inconclusive; Tollen's orcinol reaction for pentose was negative; the phloroglucinol test for lignin hexose, pentose or galacturonic acid was inconclusive and the naphthoresorcinol test for hexuronic acids was positive. Ruthenium red and alcohol precipitated a pink material which, when collected and hydrolyzed, gave a positive Benedict's test for reducing sugar. The supernatant of this precipitation gave no positive reactions for the Benedict's or Molisch's tests. The total carbohydrate was thus considered pectic material.

The unbleached supernatant gave identical results except for the biuret reaction which was inconclusive. When this supernatant was treated with trichloroacetic acid, a considerable amount of dark precipitate was produced, leaving a relatively clear solution. Collected by centrifugation, washed, neutralized, and dried, the precipitated material would not react positive to biuret or ninhydrin. The supernatant after trichloroacetic acid would not produce a positive reaction for either of these two tests. The precipitate produced a very strong Molisch's test, but it would not fix Ruthenium red dye. However, Ruthenium red plus alcohol caused a precipitate from the supernatant which gave a positive Molisch's test and a slow Benedict's test after hydrolysis. Thus, pectic materials were again demonstrated in the supernatant after ammonium oxalate digestion of teliospores.

d. *Tests on the Spore Residue of the Ammonium Oxalate Fraction.*

Initially, spores were mounted in Shear's mounting fluid (**1**) to note any alterations that had been produced by the oxalate digestion. The sheath was irregularly disorganized on most spores. There were numerous spores, however, on which the sheath appeared to be more or less intact. Upon adding Ruthenium red dye to the edge of the coverslip, there was uptake of the stain by the sheath residues, the reticulum and the endospore. Upon heating, the reticulum alone remained red,

[3] Solution was made alkaline, aerated an hour and then neutralized before ninhydrin reagent was added in order to be rid of ammonia from the oxalate digest.

showing that not all of the pectinaceous materials had been removed by the solvent or that other materials were present that fixed the dye. There was no evidence of differential dye uptake by the inner portions of the endospore wall.

The Prussian blue reaction was performed after mordanting a sample of the spores in 2% ferric chloride. The sheath residues, the reticulum and the protoplast reacted positive. There was no blue stain in the endospore nor the previously well-defined inner portion of this layer. The indications were, then, that the reaction was positive previously for a constituent dissolved by ammonium oxalate. Most likely it was pectic material.

There was less color in the reticulum than previous to the ammonium oxalate digestion. However, the structure was still strongly argentaffin, although the protoplast was inconclusively argentaffin.

The ninhydrin test [4] was still positive on the reticulum and protoplast, and the Millon's reaction was weakly positive for the reticulum. It was still inconclusive for the protoplast. The xanthoproteic test produced an orange color in the protoplast.

The lipophilic stains responded generally as they had previously. However, no free oil globules could be detected in the sample mounts, and neither Nile blue A nor Sudan black B defined the narrow band of lipophilic material between the endospore and the reticulum they had previous to the oxalate digest.

After washing spores in weakly acidified 70% ethyl alcohol, some were placed in a solution of Resorcin blue; others were placed in Methylene blue. After 24 hours they were washed in distilled water and mounted in Shear's mounting fluid. They failed to fix Resorcin blue, but there was strong uptake of Methylene blue in all parts of the spore. Upon adding 2% HCl to the edge of the coverslip of the mount with Methylene blue, color was lost from the sheath residue and the reticulum faded to a blue tinge. Hence, the dye was considered to have been fixed by both carbohydrate materials which lost the stain when acidified and nitrogenous substances which retained the stain under acid (**9**).

The Mäule reaction *per se* was negative, but the same differentiation of a double layer to the endospore was demonstrated as it had been previously when Safranin O was incorporated into the schedule. Thus, the demonstration of reducing sugars in the inner layer of the endospore

[4] Spores were aerated in weak alkali solution to remove ammonia from the oxalate digest and then neutralized before mounting in buffered (pH 7) ninhydrin solution.

included those other than from a pectic origin. The phloroglucinol test for lignin was applied histochemically. Except for slight yellowing of the reticulum, the test was negative.

The Chitosan reaction was performed on a sample of spores and the definition of a double layer to the endospore was again demonstrated. Nearly all spores had shed their reticulate layer by the treatment.

e. *The Schweitzer Supernatant.*

The supernatant of this fraction was stained a brown-black. Half of the fraction was acidified and diluted. No precipitate was formed, ruling out any appreciable amount of cellulose (**10**). The other half of the fraction was dialyzed in the refrigerator against distilled water for 48 hours. The pigment remained in the tubing. The dialysate gave positive ninhydrin and biuret reactions, but no precipitate was obtained with trichloroacetic acid. These tests were considered inconclusive on the suggestion that ammonia complexes from the Schweitzer reagent were responsible for the positive tests.

The Molisch's reaction was negative on this dialysate; so, as expected, the Benedict's test for reducing sugars and the Tauber's test for pentoses or hexoses were also negative. The phloroglucinol reaction for lignin was negative as was the iodine-potassium iodide plus sulfuric acid or zinc chloride tests for lignin, cellulose and chitin. Alcohol produced no precipitates from the dialysate. Thus, no definable pectins or hemicellulose were present.

The dialyzed sample produced a very strong Molisch's reaction, but it was negative to the Benedict's and Tauber's tests. The pigment was ether-insoluble and was precipitated from solution by one volume of 2% ferric chloride solution per two volumes of sample. The filtrate of this precipitation was negative to Molisch's, Benedict's, Tauber's, Ruthenium red and Resorcin blue tests. No precipitate was formed by trichloroacetic acid. However, the ninhydrin and biuret reactions were weakly positive as in the dialysate. The phloroglucinol and iodine-potassium iodide plus sulfuric acid or zinc chloride tests for lignins, cellulose or chitin were all negative.

The pigmented residue from the ferric chloride precipitation was re-suspended in water and brought to pH 8 with sodium hydroxide to remove excess iron. The precipitate was re-collected and tested for carbohydrates, proteins, melanin and chitin.

The Molisch's test was very strong but the Benedict's and Tauber's tests were negative. Upon acid hydrolysis the Benedict's test was weakly positive, and the Tauber's test was inconclusive. The ninhydrin and biuret reactions for proteins were applied to the precipitate histo-

chemically. The results were inconclusive, being masked by the pigments present. The fact that the pigment was precipitated by 2% ferric chloride was highly suggestive of melanin. This pigment is soluble in hot, 20% ferric chloride (**10**). The hot reagent applied to the precipitate reduced and considerably lightened it. The sample was centrifuged and the concentrated ferric chloride supernatant was decanted. One-third of it was treated with trichloroacetic acid. A brown-black precipitate was produced. Another third of the solution was diluted tenfold and cooled in the refrigerator overnight. By morning a thin film of black pigment had settled from solution. Dialyzed against running tap water to remove excess iron, the last third of the solution yielded considerable black pigment. All these characteristics agree for melanin pigment.

The lightened precipitate from the hot, concentrated ferric chloride treatment was dialyzed against running tap water overnight. A positive biuret reaction was obtained on this residue. The remaining residue was digested for thirty minutes in 40% potassium hydroxide under 15 pounds steam pressure. The chitosan reaction was completed on it and proved positive for chitin. Thus, the previous weak tests for protein and strong Molisch's reaction were probably owing in part to glucosamine.

f. *Test on the Spore Residue of the Schweitzer Fraction.*

The sample of spore residue from this fraction was leached in several changes of 5% acetic acid to be rid of ammoniacal copper hydroxide. It was then made alkaline with sodium hydroxide and warmed to evolve last traces of ammonia. The sample was washed in several changes of distilled water and tested histochemically as in previous fractions.

The remnants of sheath, the reticulum and the protoplast fixed Ruthenium red dye, and produced a positive Prussian blue reaction. However, only the reticulum remained red after heating Ruthenium red mounts, indicating pectic materials there only (**9**). Both treatments caused the residual sheath and reticulum to become appressed to the endospore wall. Resorcin blue dye was lightly fixed in the reticulum of all spores. The protoplasts remained colorless in mature spores, but were stained blue in immature spores and sterile cells.

Very little color remained in the spore walls. It was only weakly argentaffin in the reticulum. There was no silver reduced in the protoplast. The ninhydrin reaction was faintly positive in the protoplast. The biuret reaction was highly caustic and inconclusive. Lipophilic stains were fixed only in protoplast oil globules except for Nile blue, which was also fixed in the reticulum. The chitosan reaction reduced

the spore residue to a more or less amorphous state in which the skeletons of the endospores could be defined as chitinous rings.

The phloroglucinol reaction was performed histochemically on spores. Except for a light yellowing, the test was negative.

g. *The Second Ammonium Oxalate Supernatant.*

The supernatant was colored green. This was considered due to the copper ions still present from the previous treatment with Schweitzer's reagent. The Molisch's test was strongly positive and when two volumes of alcohol were added to one volume of the supernatant, a whitish precipitate was formed. This precipitate readily dissolved in distilled water and was collected as a red precipitate when two volumes of alcohol containing Ruthenium red were added to one volume of the solution. The Benedict's test was negative on the original supernatant, but was positive after a long time on the precipitate produced by alcohol. Trichloroacetic acid turned the original supernatant cloudy, but produced no precipitate. The biuret reaction was negative, although the ninhydrin reaction was positive. The latter reaction was considered inconclusive, owing to probable ammonia ions released from the traces of oxalate or Schweitzer's reagents still present. Resorcin blue in alcohol caused no blue precipitate to be formed. Two per cent ferric chloride produced a brown black precipitate which tested positive for melanin. It did not test positive for chitin.

h. *Tests on the Spore Residue of the Second Ammonium Oxalate.*

Spores were nearly void of sheath material and fixed Ruthenium red throughout. Upon heating, the stain disappeared from all structures but the reticulum. Even there the color was considerably faded. The Prussian blue reaction was positive in all structures. Resorcin blue dye was still fixed in walls and protoplasts of immature spores and sterile cells.

Other histologic tests were abandoned as residual ions from the digests confused the interpretations, or they were too caustic for suitable differentiation.

i. *The Alcohol and Ether Supernatants.*

This fraction was expected to contain lipoidal materials. It was evaporated to remove solvents, mixed with 95% alcohol and evaporated to a paste twice to remove water, and finally extracted with fresh ether. Upon standing, a transparent ether layer was produced atop a cloudy layer below. The upper layer was drawn off for testing.

The ether was distilled off, leaving a dark brown oily material. This was treated with four volumes of reagent grade acetone. A milky solution was produced which, upon swirling, caused a precipitate. The pre-

cipitate was collected by centrifugation and the two fractions were separated and tested.

The precipitate portion turned concentrated sulfuric acid yellow and was turned a mahogany brown after treatment with iodine-potassium iodide and hydrochloric acid. These tests indicated the material to be a phospholipid.

Some of the precipitate was dissolved in 10 cc 95% alcohol and heated to boiling over a hot water bath. The material was hydrolyzed with 10 cc concentrated hydrochloric acid for 20 minutes over boiling water. After hydrolysis the Benedict's test was performed. It was negative for reducing sugar, so the phospholipid was not a glycolipid.

The remaining hydrolysate was tested for glycerine, fatty acids and phosphorus according to standard procedures (**10**). All were present, definitely showing the material to be phospholipid in nature. Identification of the fatty acids was not attempted.

The supernatant fraction of the acetone precipitation was evaporated to dryness. A portion was redissolved in chloroform and the Salkowski's reaction for cholesterol and ergosterol was run. A dark red ring was produced, including the greenish fluorescence of the upper layer typical of ergosterol.

Upon acid hydrolysis of the supernatant fraction of the acetone precipitation above, the Benedict's test was again run. The test was negative. Hence, there was no glycolipid in the fraction. After extractions with solvents and saponifying according to standard procedures (**10**), the acrolein test for glycerine proved positive and fatty acids were present. However, the molybdate test for phosphorus was negative, showing the absence of any phospholipid in the fraction.

j. *Tests on the Spore Residue of the Alcohol-ether Fraction.*

Spores appeared the same as those from the previous fraction, except that Ruthenium red dye remained fixed after heating in the residual sheath as well as in the reticulum. Resorcin blue was still fixed throughout.

k. *The Alcoholic Potash Supernatant.*

The fraction was dried and then treated with ether to remove nonsaponifiable lipids. The residue was then dissolved completely in hot distilled water and neutralized with dilute acid. The Molisch's test for carbohydrates, and the Benedict's test for reducing sugars were both positive. The Tauber's test was positive for hexose.

A pink precipitate was produced by alcohol containing Ruthenium red dye. A bluish-white precipitate was produced by alcohol containing Resorcin blue dye, and a bright blue precipitate was obtained by alcohol

containing Methylene blue dye that lost color upon the addition of weak acid.

After acid hydrolysis of the hot water extract with 6N sulfuric acid, tests for glycerol were positive (acrolein test), and fatty acids were obtained. Tests for proteins or their derivatives were all negative.

1. *Tests on the Spore Residue of the Alcoholic Potash Fraction.*

After neutralizing spores with weak acid, the reticulations of spores in microscopic mounts were free from sheath residue and had lost rigidity. They were somewhat amorphous. They would not fix Ruthenium red or Resorcin blue dyes. They readily fixed Methylene blue dye throughout, but lost all color when 2% hydrochloric acid was introduced at the edge of the coverslip. Spores failed to produce a ninhydrin reaction histochemically, but a sample of spores hydrolyzed in 40% sodium hydroxide gave a strong biuret reaction. A sample of spores also gave a strong Molisch's reaction, and upon hydrolysis gave a strong Benedict's test. The residual reticulum was therefore considered to contain protein and unidentified hemicelluloses, as pectic materials, certain hemicellulose fractions including callose, and cellulose would have been removed in the previous extractions.

B. *Enzymatic Digestions on Teliospores*

1. *Procedure.*

A sample of dwarf bunt teliospores was leached in distilled water overnight at room temperature over a magnestirrer, collected and then refluxed for one hour in 95% alcohol over boiling water. After collecting and desiccating under low vacuum, spores were leached for 2 hours in ether. The ether leaching was repeated twice. Spores were collected and again placed under vacuum to remove solvent. The dried residue was separated into four parts for enzymatic digestions.

One part was placed in pectinase enzyme (pectin-polygalacturonase)[5] buffered at pH 4.5 and incubated at 37° C for 36 hours; one part was placed in hemicellulase enzyme[5] buffered at pH 4.5 and incubated at 37° C for 36 hours; one part was placed in cellulase enzyme[6] buffered at pH 5.3 and incubated at 37° C for 36 hours; and one part was placed in lysozyme enzyme[5] buffered at pH 5.3 and incubated at 25° C for 36 hours. Spores were washed in several changes of pH 7 phosphate buffer

[5] Commercial enzymes obtained from Nutritional Biochemical Corporation, Cleveland, Ohio.

[6] Cellulase 36 Concentration, factor 3.7, obtained from Rohm and Haas Co., Philadelphia, Pa.

to remove enzymes and microscopic mounts were then made to observe alterations of spore morphology. Mounts of spores were also made after treating them with Resorcin blue and Ruthenium red dyes according to standard procedures (**16**, **18**).

2. *Results.*

a. *Pectinase Digestion.*

Spores mounted in Shear's mounting fluid were devoid of a definable sheath, but free, irregular masses of a granular matrix could be seen adjacent to and adhering to spores. After 18 hours in 1:1000 Resorcin blue dye, mature spores and the granular matrix had failed to fix the dye. However, sterile cells and very immature spores had fixed the dye strongly. In the stained cells, walls were stained a light blue and the protoplast region, including a clear region surrounding the plasmolyzed protoplast, stained an intense blue. Spores placed in Ruthenium red dye (1:5000, in distilled water) had absorbed the dye intensely in the reticulum and endospore regions. The background granular matrix also was stained pink. Protoplasts of mature spores were colorless, but those of sterile cells were stained red, and those of immature spores were stained pink. Upon heating the mounts, the red stain disappeared from all structures except the protoplasts of immature spores and sterile cells. Thus, the uptake of the dye was considered to have been effected by non-uronide carboxyl-containing compounds (**9**) in the wall structures.

b. *Hemicellulase and Cellulase Digestions.*

Spores mounted in Shear's mounting fluid were as devoid of a definite sheath as the pectinase digestions. They responded to Ruthenium red and Resorcin blue dyes the same as after pectinase digestion. Since these enzymes are notably impure, the hydroxamic acid reaction of McComb and McCready (**11**) to assay pectase (Pectinesterase) and the pectinase assay method of Reid (**17**) were performed. Both enzymes produced large clear zones on the media in these tests. Thus, the two digestions on teliospores were considered inconclusive.

c. *Lysozyme Digestion.*

Spores mounted in Shear's mounting fluid from this digestion had well defined sheaths. There was no granular matrix in mounts. The endospore was much thicker on spores after this digest than after the other digests. Resorcin blue dye was strongly fixed in sheath material. The reticular layer was mostly unstained on what were alleged to be mature spores. The endospore layer of all such spores did not fix the dye. However, obviously immature spores fixed the dye in all wall layers, and in their protoplasts. Sterile cells fixed the dye throughout as well. Ruthenium red dye was fixed in all wall parts. It was also

fixed in protoplasts of immature spores and sterile cells. Upon heating, the stain disappeared from the endospore layer except for a light pink band proximal to the protoplast. The reticulum remained red and the sheath was considerably faded.

C. *Chemical and Mechanical Separation of Wall Parts*

In various atempts to dissolve wall parts the usual solvents for proteins, pectic substances and various carbohydrates were employed. From the standpoint of gross structure separations, sheath was completely dissolved in 5% alcoholic potash after an hour's reflux over boiling water. There was no apparent morphologic change in other wall components except for swelling. Hot 10% sodium hypochlorite and cold *Eau de Javelle* (potassium hypochlorite) (12) dissolved both sheaths and reticulae. If spores were mounted in 5% potassium hydroxide and 5% sodium hypochlorite was added to the edge of the coverslip, both the sheath and the reticulum were observed to dissolve from spores (FIG. 1F). Spores mounted in Shear's mounting fluid and treated with cold 5% sodium hypochlorite could be observed to expand the reticulum away from the endospore, appearing similar to the spore in FIG. 1A. Neither the sheath nor the reticulum of spores was dissolved unless the mount was heated.

In reversing the procedure for dissolving sheath and reticulum, i.e., treating spores with sodium hypochlorite (using a 1% rather than a 5% solution) followed by potassium hydroxide, the sheath dissolved and the reticulum was expanded away from the endospore. Spores then rinsed and mounted in distilled water exhibited oil globules partially surrounding the endospores inside the expanded reticulum (FIG. 1E). Crystal violet, Thionin, Safranin O, Ruthenium red, Phloxine and Rose bengal dyes all caused the expanded reticulum to contract again about the endospore in these mounts.

For better definition of wall parts, Safranin O dye was used to stain spores prior to the above oxidation schedule. The color changed to shades of orange, but was not completely removed by the oxidation treatment. Furthermore, it did not prevent expansion of the reticulum away from the endospore, or mask the oil globules. Two layers were evident to the endospore (FIG. 1A). No oil globules were observed in spores after the oxidation schedule when they had been previously refluxed in alcoholic potash or when they had been previously leached in ether (FIG. 1B).

Since the protoplast usually was plasmolyzed by the sodium hypo-

chlorite and interfered with observations on mechanical separation attempts, spores were placed in Navaschin's fixative (**12**) for 72 hours prior to the oxidation schedule to fix the proteinaceous elements of the spore. After fixation, spores were washed in water and then oxidized in 1% sodium hypochlorite for 3 minutes. They were then transferred

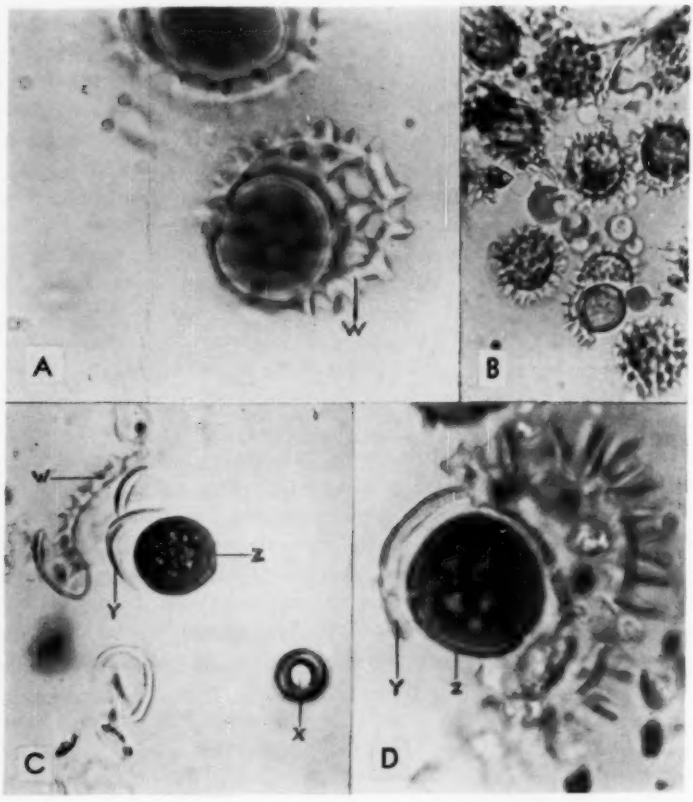

FIG. 2. Mechanical separation of wall layers of fixed spores after 72 hours in Shear's mounting fluid when oxidized first with NaClO followed by KOH and then stained with Safranin O. A. Removal of the reticulum (w) with slight agitation to the coverslip. B. Releasing the lipoid containing viscous globule (x) with slight pressure and agitation. C. Fracturing loose the outer endospore layer (y) with increased pressure, leaving the protoplast surrounded by a thin inner endospore layer (z). D. The crushed inner endospore layer and protoplast after very heavy pressure.

to 3% potassium hydroxide for 15 minutes. After washing them several times in distilled water over a period of an hour to remove reagents, spores were stained for 15 minutes in Safranin O dye in water. Upon mounting spores in Shear's mounting fluid, initial attempts to crush spores to separate layers were difficult and inconclusive. However, if spore mounts were allowed to stand for 72 hours or longer, the spores were easily separated mechanically into distinct morphological components (FIG. 2).

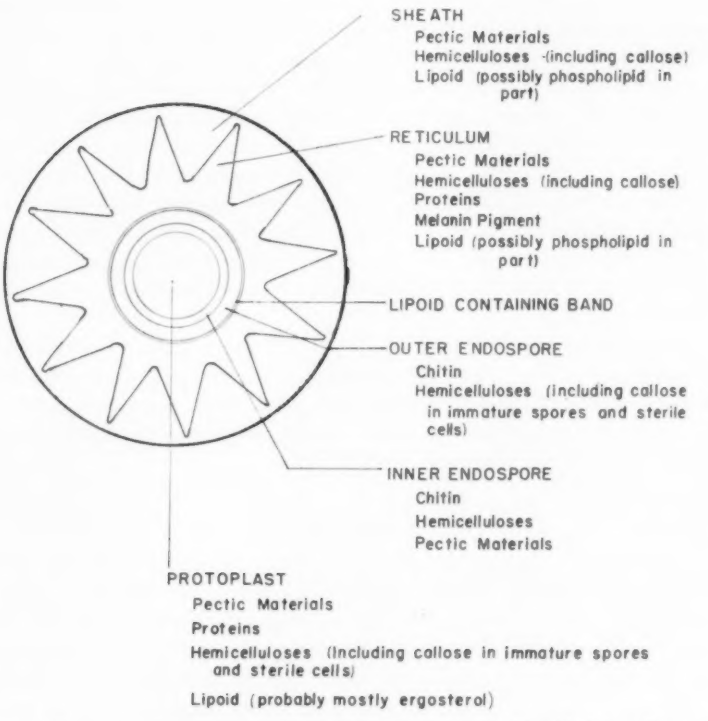

FIG. 3. Graphic summation of some chemical constituents identified in teliospore layers of the dwarf bunt fungus.

The reticulum separated from spores with a very light pressure of a dissecting needle on the coverslip (FIG. 2A). A little heavier pressure released the oily globule adhering to the surface of the endospore (FIG. 2B). Still heavier pressure fractured loose an outer layer of the

endospore wall, leaving the protoplast surrounded by a thin but distinct layer (FIG. 2C). Very heavy pressure cracked the proximal layer to the protoplast and the fixed protoplast (FIG. 2D).

D. *Discussion and Conclusions*

As elucidated by the foregoing procedures and digestions on *Tilletia contraversa,* this study substantiates the thesis of van Wisselingh (**21**) that the teliospore walls of *Tilletia* possess three distinct layers. The layers are separable mechanically and are exclusive of a pectinaceous sheath component which is characteristic of the particular test organism employed. In addition, the test organism was demonstrated to incorporate within its context of the three layers a thin, viscous, lipoid-containing matrix located between the reticulum and the endospore. As shown in a previous study (**7**), this matrix was defined by certain lipophilic dyes. Because it was mechanically separable after strong oxidation treatments in these studies, the evidence strongly indicates that it may be considered a distinct layer. It is suggested that the material may function as an agent which cements the reticulum to the endospore. Such being the case, the term "mesospore" might be employed for want of a better one.

The diverse tests employed show that the layers defined were different in chemical context. Each layer possessed its own combination of identified materials. FIGURE 3 graphically summarizes the above study on the constituents identified and the mechanically separable layers of the dwarf bunt fungus teliospore.

SUMMARY

Employing various reagents to dissolve or to digest portions of the teliospore of *Tilletia contraversa* Kühn in conjunction with histochemical procedures, the morphology of the spore was determined and the parts were separated mechanically. The teliospore wall consists of several distinct layers, incorporating a sheath, a reticulum and a two-layered endospore. The reticulum is cemented to the endospore by a lipoid-containing material.

By chemical tests and histochemical techniques, the chemical composition of the various layers was defined in part. The sheath was primarily a pectic material complex, incorporating hemicelluloses (including callose) and lipoids (possibly phospholipid in part). The reticulum contained pectic materials, hemicelluloses (including callose), proteins, melanin pigments and lipoids (possibly phospholipid in part). The

material cementing the reticulum to the endospore contained lipoids. Other constituents of the material could not be defined as separate from other layer materials, although chitin was indicated. The outer endospore layer was primarily chitin, but some hemicelluloses (including callose in immature spores and sterile cells) were defined histochemically. The inner endospore layer contained at least chitin, hemicelluloses and pectic materials. Callose and protein were probably present but were not distinct from adjacent structures in the tests employed. The protoplast contained pectic materials, proteins, hemicelluloses (including callose in immature spores and sterile cells) and lipoids (mostly ergosterol).

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LITERATURE CITED

1. **Chupp, C.** 1940. Further notes on double cover-glass mounts. Mycologia **32**: 269–270.
2. **Conn, H. J.** 1953. Biological stains. 6th Ed. 367 pp. The Williams and Wilkins Co., Baltimore, Md.
3. **Fishman, W. H.** 1951. Enzymatic hydrolysis of micropolysaccharides. Chap. 22. (*In:* The Enzymes. Vol. 1. Part II. Ed. Sumner and Myrbäck.) The Academic Press, Inc. New York, N. Y.
4. **Glick, David.** 1949. Techniques in histo- and cytochemistry. 531 pp. Interscience Publishers, Inc. New York, N. Y.
5. **Gomori, George.** 1952. Microscopic histochemistry. 273 pp. University of Chicago Press. Chicago, Ill.
6. **Graham, S. O.** 1957. The morphology and chemistry of the teliospore wall of the dwarf bunt organism. Phytopathology **47**: 522. (Abstract.)
7. **Graham, S. O.** 1959. The effects of various reagents, mounting media, and dyes on the teliospore walls of *Tilletia contraversa* Kühn. Mycologia **51**: 477–491. [1961.]
8. **Kaufmann, C., and E. Lehmann.** 1926. Kritische Untersuchungen über die Spezifitatsbreite histochemischer Fettdifferenzierungsmethoden. Centralbl. für Allg. Path. **37**: 147–152.
9. **Kertesz, Z. I.** 1951. The pectic substances. 628 pp. Interscience Publishers, Inc. New York, N. Y.
10. **Koch, F. C., and M. E. Hanke.** 1953. Practical methods in biochemistry. 6th Ed. 537 pp. The Williams and Wilkins Co., Baltimore, Md.
11. **McComb, E. A., and R. M. McCready.** 1958. Use of hydroxamic acid reaction for determining pectinesterase activity. Stain Tech. **33**: 129–131.
12. **McLean, R. C., and W. R. I. Cook.** 1941. Plant science formulae. 203 pp. MacMillan and Co., Ltd. London, England.
13. **Meiners, J. P.** 1954. Histochemical studies on differences in rate of germination of common and dwarf bunt spores. Phytopathology **44**: 498. (Abstract.)

14. **Pigman, W. W., and R. M. Goepp, Jr.** 1948. Chemistry of the carbohydrates. 748 pp. The Academic Press, Inc. New York, N. Y.
15. **Pigman, W. W.** 1951. Cellulases, hemicellulases and related enzymes. Chap. 20. (*In:* The Enyzymes. Vol. I. Part II. Ed. Sumner and Myrbäck.) The Academic Press, Inc. New York, N. Y.
16. **Rawlins, T. E., and W. N. Takahashi.** 1952. Techniques of plant histochemistry and virology. 125 pp. The National Press. Milbrae, Calif.
17. **Reid, W. W.** 1950. Estimation and separation of the pectin esterase and polygalacturonase of micro-fungi. Nature **166**: 569.
18. **Thomas, R. C.** 1928. Composition of fungus hyphae. I. The Fusaria. Am. Jour. Bot. **15**: 537–547.
19. **Thomas, R. C.** 1930. Composition of fungus hyphae. II. Sclerotinia. Am. Jour. Bot. **17**: 779–788.
20. **Thomas, R. C.** 1942. Composition of fungus hyphae. III. The Pythiaceae. Ohio Jour. Sci. **42**: 60–62.
21. **Wisselingh, C. van.** 1898. Mikrochemische untersuchungen über die Zellwande der Fungi. Jahrb. Wiss. Botan. **31**: 619–687.

THE SYSTEMATIC POSITION OF THE MYXOMYCETES [1]

G. W. Martin

In the long history of the attempt to classify living organisms the limits and status of many groups have undergone great modification, but surely there are few of them in which the changes have been greater than in the Myxomycetes. Originally studied by botanists, and particularly by botanists interested in fungi, they have for nearly a century confidently been included in the Protozoa by zoologists and, with somewhat less confidence, in the fungi by botanists. The limits of the group so designated have fluctuated widely; the supposed relationships of those included, with each other and with those excluded, have been variously interpreted and the classification within the group itself has been inconstant. The detailed study of the Myxomycetes has, it is true, been very largely left to mycologists, and the extensive literature concerning them, particularly that which deals with their classification, is to be found mainly in botanical books and journals. That proves nothing about relationships, but it does suggest that any bias toward a particular opinion about their systematic position should be guarded against with particular care by anyone representing a particular field who attempts to examine the evidence on the subject.

It is probably too much to expect that when one has come to certain conclusions after careful study and has defended them publicly, he will lightly abandon them. I have on various occasions (**1932**, **1940**, **1955**) published at length my views on the subject I am to discuss to-day. With every desire to be objective, I cannot hope that I shall be able to overcome all such bias. To what extent I shall succeed, I must let others judge.

Classification is inexorably progressive, sometimes, it seems, despite the desire of its practitioners. But it is also inevitably tied to previous work as that, in its turn, has from time to time been modified by new facts or supposed facts. For that reason, it seems desirable to summarize very briefly some of the more important steps in the classification of the Myxomycetes.

[1] Presented at a symposium of the Mycological Society of America on "Classification of Fungus Groups of Debatable Affinity" at the Chicago meeting of the A. A. A. S., December 30, 1959.

Early studies were devoted to descriptions of genera and species. These culminated in the publication of Persoon's *Synopsis Methodica Fungorum* (**1801**), in which 77 species in 11 genera, comprising a substantial majority of the commoner species, were listed. Persoon classified them as Gasteromycetes, as had his predecessors. Fries (**1829**) segregated them as a distinct group, his Suborder Myxogastres, separated on the basis of the plasmodium from the other Gasteromycetes. Link (**1833**) used the name Myxomycetes for the group, separating it from the Gasteromycetes and treating the two as coordinate suborders. With this modification, Fries's excellent treatment remained the standard for a generation.

DeBary's most important publications on the slime molds appeared from 1858 to 1864. In the first of this series of notable papers (**1858**) he announced his conclusion that these organisms were not to be included in the fungi but should be transferred to the animal kingdom as the Mycetozoa. In the following year (**1859**) he presented the results of his studies in greater detail, choosing a zoological journal and using in his subtitle the phrase "die niedersten Thiere." This view was immediately attacked. He defended it in 1862 and again in 1864, the latter being what he regarded as the second edition of his 1859 study, but with greatly altered text and the subtitle reading "die niedersten Organismen." In this book he specifically says "die Grenzlinie zwischen die niedersten Regionen beider Reiche überhaupt nur wenig scharf gezogen werden kann" and suggests that the matter is not of great importance. While he was not greatly interested in taxonomy, shifting the Myxomycetes from the fungi to the animals was a major taxonomic decision, starting a controversy which has not yet been settled but which has come to seem less important to-day than it did a century ago.

DeBary turned over the taxonomic studies to his student Rostafinski, who presented a doctor's dissertation, summarized in the *Versuch* (**1873**), in which he proposed significant changes in the currently accepted Friesian classification and made *Ceratium* the sole representative of his Cohort Exosporeae, grouping the others, including *Dictyostelium*, in the Endosporeae. In the *Monograph* (**1874–1876**), written in Polish, he established the essential basis for most of the subsequent classifications by botanists, just as the very greatly enlarged concept of its circumscription proposed by Zopf (**1885**) and Lankester (**1885**) has had great influence with zoologists. Lankester is very emphatic about the absurdity of assigning the Mycetozoa anywhere except to the animal kingdom.

Since 1885, a number of taxonomic treatments of the Myxomycetes

have appeared. Nearly all recognize the Exosporeae and Endosporeae. Schroeter (**1885–1886**) included the Acrasieae, Myxogastres and Plasmodiophorales as orders. In 1889, he made them classes, suggesting, but not formally proposing, that the forms inserted by Zopf and others be made another class, Myxozoa, to be united with the Myxophyta as a super-group, Myxobia. Only Massee (**1892**) rejected *Ceratiomyxa*. The various editions of the Lister (**1894, 1911, 1925**) and Macbride (**1899, 1922, 1934**) monographs are sufficiently familiar to require little comment. Their treatment is essentially the same, except for the inclusion of the Plasmodiophorales in the first two editions of Macbride. Schinz (**1920**) follows the second edition of Lister very closely; Hagelstein (**1944**) the third.

Jahn (**1928**), in the second edition of Engler and Prantl, divided the Myxomycetes into nine orders, of which the first, the Hydromyxales, included some of the forms which Zopf had added to the group, the second was the Exosporeae and the others comprised the Endosporeae. Tippo (**1942**) made the Myxomycophyta the 11th phylum of his series, without defining its scope, but by making the Eumycophyta his 12th phylum he indicated sharp separation of the two.

Martin (**1949**) treated the Myxomycetes as a class of the Fungi, coordinate with the Phycomycetes, Ascomycetes and Basidiomycetes, restricting the class to the two long-recognized subclasses Exosporeae and Endosporeae, with one order in the former and four in the latter. The only important changes in classification were in the sequence of orders and families and in the suppression of some families. It has long been a convention in taxonomy that each sequence shall start with the least-specialized representatives and proceed to those more specialized and theoretically more highly evolved. Bold (**1957**) raised the group to the rank of a division, as the Myxomycota, coordinate with the Phycomycota, Ascomycota and Basidiomycota, dividing it into the three classes Myxomycetes, Acrasiomycetes and Plasmodiophoromycetes.

As has been seen, the inclusion of the Myxomycetes among the Fungi and, more specifically, the Gasteromycetes, was not questioned until the time of deBary, who in 1858 relegated them definitely to the Protozoa. By 1864 he had become much less positive and noted that at the simpler levels the distinction between plants and animals is obscure. In his thesis of 1873, written under deBary's direction, Rostafinski says at the end, in emphasized type: "Die Mycetozoen sind ebenso den Pilzen wie den ächten Thieren verwandt!" Ever since, the zoologists have included the Mycetozoa in the Protozoa, while writers of botanical and mycological texts have expressed many reservations about including the

Myxomycetes within their areas. The limits have been as narrow as the Endosporeae alone and have been widened to include the Acrasieae, the Plasmodiophorales, the Labyrinthulales and a host of rhizopods. They have been classed as a subfamily or family all the way up to a division. There is still uncertainty about which characters are of prime importance, the zoologists, in general, stressing the assimilative phase, the mycologists, the reproductive.

These doubts and uncertainties are easily understandable. If it is assumed that all organisms must be plants or animals, the arguments for classifying the Mycetozoa with the Protozoa are very strong. I have recently reviewed this subject (**1955**) and shall not repeat other than to say that the available evidence makes it seem plausible that the fungi, with a few possible exceptions, have originated from colorless flagellates and are not related to the green plants. It is quite possible to find in current work many other expressed doubts about this long-familiar assumption. Moreau (**1954**) quite definitely derives the Fungi from zooflagellates and related forms even though he does exclude the Myxomycetes from them. Copeland (**1956**) recognized four kingdoms of organisms, restricting the plant kingdom to the green algae and embryophytes and the animal kingdom to multicellular forms. He erects the Kingdom Mychota for the bacteria and blue-green algae and the Kingdom Protoctista for the Protozoa, Fungi and all algae but green algae. The bulk of the Fungi are included in the Phylum Inophyta and a smaller number to two other phyla, while the Mycetozoa, including the Plasmodiophorales, are assigned to the Protoplasta, between the Zoomastigoda and Rhizopoda, hence definitely associated with traditional animal groups.

There are, then, two problems to consider. First, what are the limits of the Myxomycetes or Mycetozoa as a natural group? Second, what is to be their taxonomic position? In attempting to answer these questions I realize that it must be on the basis of incomplete information and that any answers I attempt are at best opinions formulated on the basis of long, but not necessarily profound, study.

The relation of *Ceratiomyxa* to the Myxomycetes has never been seriously questioned since the appearance of the beautiful study by Famintzen and Woronin (**1873**). In the year it was published, Rostafinski erected the Subclass Exosporeae for the genus. Even earlier, the Acrasieae had been included in the Myxomycetes, and later the Plasmodiophorales, the Labyrinthulales and a number of Protozoa were added.

So far as the Labyrinthulales are concerned, there seems to be little doubt that any resemblance they have to the Myxomycetes is purely

superficial and that their original inclusion was due to a complete misunderstanding of the nature of the so-called net plasmodium. The case of the Monadineae of Zopf is somewhat less clear, but current information suggests that the resemblances between them and the Myxomycetes are not sufficiently fundamental to justify the assumption of more than a collateral relationship. For the present, the Labyrinthulales certainly, and the Monadineae almost as certainly, may best be relegated to that side of a vague and indeterminable boundary which is the recognized province of the protozoologists.

The Acrasieae present only slightly greater difficulty. The active assimilative stage is represented by the free amoebae and the pseudoplasmodium is really a part of the reproductive stage, the individuality of the cells composing it being maintained throughout the development of the sporocarp. There are no flagellated cells and a diploid stage, if it exists at all, is extremely transitory. If they are to be retained among the fungi, it must be partly as a matter of convenience and certainly not in any way that suggests close relationship with the Myxomycetes. There is an excellent survey of these and related questions in the recent book by Bonner (**1959**).[2]

The Plasmodiophorales have been shifted from the Myxomycetes to the Phycomycetes and back again for many years. The multinucleate mass of protoplasm filling host cells and sometimes connected by slender protoplasmic strands with similar masses in adjoining cells certainly suggests a plasmodium. The discovery by Ledingham (**1934**) that the swarm-cells were biflagellate seemed at the time to remove them from the Myxomycetes, which were then supposed to have uniflagellate swarm-cells. The demonstration by Elliott (**1949**) that the swarm-cells of the Myxomycetes are really biflagellate apparently reversed this decision.[3]

[2] Bonner regards the Mycetozoa as a useful assemblage of four groups, Myxomycetales, Plasmodiophorales, Labyrinthulales and Acrasiales, which "probably have little or no relation one to another." This is to make of them a form class, which I do not think is at the present time an acceptable taxonomic disposition.

[3] Recently, question has been raised concerning the biflagellate condition of the swarm-cells. At the Montreal Congress, A. L. Cohen presented a significant paper, illustrated with remarkable electron-microscope photographs, in which he claimed that swarm-cells of Myxomycetes may be either uniflagellate or biflagellate and that sometimes swarm-cells secured by germinating spores from the same fructification may be of both sorts. Furthermore, he showed that the pseudopodia often observed at the posterior end of a swarm-cell may originate at the anterior end as cylindrical protrusions adjacent to the long flagellum and migrate toward the posterior end, and suggested that such pseudopodia may have been responsible for some of the reports of a second flagellum. Some years ago, I found that by placing a droplet containing living swarm-cells on a slide and by dropping on them with

However, the marked difference in life cycle, as at present understood, still presents a barrier. In his 1959 paper, Sparrow suggested that the Plasmodiophorales be made the sole order of a new Class Plasmodiophoromycetes. This may prove to be the best disposition of the group.

As thus restricted to the Exosporeae and the Endosporeae, the Myxomycetes becomes a coherent class. In earlier treatments, I have regarded them as constituting a class of the Fungi, not necessarily the lowest, since some of the chytrids are certainly much simpler in organization, but conveniently put first in the series because they have retained more of the protozoan characters of the ancestral forms I have postulated for the fungi than have the other classes. There can be no doubt that the swarm-cells and myxamoebae and presumably the plasmodia are able to ingest bacteria, algae and fungous spores and digest them in animal-like fashion. It has been assumed for many years that at least part of their nutrition in the plasmodial stage has been by nutrients in solution, but until recently attempts to grow them in pure culture have been inconclusive. Recent work, particularly that of Cohen (**1939**), Daniel and Rusch (**1956**) and some unreported studies by W. R. Lazo, has shown that plasmodia completely freed from bacteria may grow vigorously on both solid and in liquid media; in other words, their nutrition under these conditions is entirely fungus-like. This greatly strengthens the presumption that it may be at least partly so in nature.

Certain chytrids which cause gall-like swellings in various species of the Saprolegniaceae possess at first naked thalli which show amoeboid movement, as was first pointed out long ago by Cornu (**1872**). I have myself observed this in an *Olpidiopsis* parasitizing an *Achlya*. Even more striking is the case of *Coelomomyces* (Couch, **1945**), a genus referred on excellent grounds to the Blastocladiales. The species of

a pipette held two or three inches above the slide a drop of diluted Gram's iodine solution, so as to kill them instantly, it was possible to stain the flagella. A cover slip was then placed over the preparation and the slide examined under a high-power dry objective. Only a small proportion of the swarm-cells in such mounts showed the second flagellum. But by isolating a swarm-cell which did not show it, and tapping very lightly on the cover-glass with a fine needle, it was possible *in every case* to demonstrate the second flagellum. Elliott repeated this many times, and since then Alexopoulos and others have done the same, always with the same results. Despite the evidence of superior technique evidenced in Cohen's photographs, I cannot but believe that the violent treatment inevitable in the preparation of material for photographing by electron microscopy has in some way obscured the second flagellum in many of the swarm-cells prepared in this way. On the basis of Elliott's careful studies and the repeated observations of myself and others, I am convinced that the biflagellate condition is as constant in the Myxomycetes as it is in the biflagellate Phycomycete groups.

Coelomomyces occur within aquatic insect larvae and the mycelium is without a wall and has, indeed, been referred to as a plasmodium. There is no suggestion that either the chytrids or *Coelomomyces* are closely related to the Myxomycetes, but their inclusion in other groups of fungi does suggest that lack of hyphal walls, even if it were absolute, need not exclude the Myxomycetes from the Fungi. And it is by no means certain that it is absolute. Many years ago, Massee (**1892**) pointed out resemblances between the membrane surrounding the veins of a plasmodium and the hyphal walls of the larger mucors and later (**1932**) I enlarged on this, pointing out that one of the larger veins of such a plasmodium may, with suitable care, be lifted in its entirety from its substratum and be dropped back uninjured. The collapsed walls of such empty veins are a common sight on dead wood and leaves in a forest.

The Fungi, regarded as a taxonomic group, are much more difficult to define than, for example, the Embryophyta, and this difficulty is almost equally great whether the Myxomycetes are excluded or included. After I had completed the manuscript of the revision of the Myxomycetes for North American Flora, I was asked to write a general diagnosis of the Fungi. After much effort, I came out with a brief paragraph with which I was, and am, greatly dissatisfied. Because it tries to take into account the extraordinary variation found within the group, it fails to say very much that is definite. Such a failure may be an indication that the group is really not homogeneous. The same problem appears, in more restricted form, in an attempt to write a diagnosis of the Phycomycetes. Sparrow's proposed new classification of the Oomycetes (**1959**) is an interesting case in point. Sparrow holds that the Oomycetes comprise four distinct development series from different origins, each of which he proposes shall be given class rank to emphasize its separation from the others. Similarly, the Myxomycetes seem to represent a specialized line from the same ancestral reservoir, and to have developed a quite unique way of life.

It has often been suggested that the Myxomycetes are extremely primitive. As I have noted previously, this is not necessarily the case. The plasmodium has been shown to be able to pass, nuclei and all, through the pores of a Berkefeld filter (Moore, **1933**) and this can well explain its peculiar and specialized ability to move through the pores of wood, not to mention the interstices of leaves and soil, all of which are common habitats. As Moreau (**1954**) puts it, their simplicity is less real than appears at first sight. Their ancestry is in great doubt, other than that it appears to have been somewhere in the same complex that may have given rise to the other fungi, and they seem to have reached

the end of their particular developmental path. If this is true, they are, at their level, relatively advanced organisms.

The solution I am currently adopting is to rank the Fungi as a division, recognizing two subdivisions, the Myxomycotina, to include the single class Myxomycetes, and the Eumycotina, to include all the other classes. This expresses my conviction that there is no fundamental biological reason why the Myxomycetes, when properly restricted, should be separated from the other fungi, with which they have a long association. And if the zoologists prefer to insert them in the Protozoa, with which group affinity can also reasonably be postulated, that does less violence to the facts as we know them than the inclusion in that phylum of such genera as *Chlamydomonas, Pandorina* and *Volvox,* while many other groups—the dinoflagellates, for example—fit equally well or ill into either. Such overlapping merely emphasizes, what is now all but universally recognized, that whatever validity the terms plant and animal may possess at higher levels, they are essentially meaningless when applied to the organisms under discussion.

Within the Myxomycetes, as here restricted, the differences between *Ceratiomyxa* and all the other genera are so pronounced that ever since the genus was recognized as a myxomycete it has been felt necessary by most monographers to indicate a marked separation between it and the other genera, usually by making it the sole representative of a supergeneric category, the Exosporeae. A. Lister, in 1894, favored the view that the spore-bearing branches of *Ceratiomyxa* were homologous with the sporangia of the Endosporeae, but mentioned, without reference, two other possibilities which had been suggested, one of which was that each so-called spore with its stalk may be the equivalent of such a sporangium, and that the base and erect, often branched, structures which bear the spores constitute extensions of a complex hypothallus. G. Lister (**1925**), in the third edition of the *Monograph,* definitely accepts the homology between the sporophore as a whole and a sporangium, basing her conclusions largely upon the cytological findings of Jahn (**1908**). H. C. Gilbert (**1935**) failed to confirm Jahn's findings, showing that the primary "spore" nucleus is diploid and that the first division in the mature spore is meiotic. For this reason, and others, he concluded that each spore really is the equivalent of a sporangium and that the prominent limy base and branches really do correspond with a hypothallus. I saw Gilbert's preparations and have the strongest possible reason to believe his interpretation is correct but his findings seem to me to emphasize rather than to diminish, as he thought, the differences between the Exosporeae and the Endosporeae. The traditional separa-

tion of the two, as well as their affinity, may be satisfactorily recognized by continuing to designate them as subclasses, to be called, in accordance with the recommendations of the current botanical Code, the Ceratiomyxomycetidae and the Myxogastromycetidae. These terms are, I must confess, clumsy and inconveniently long, but after struggling to determine what the older writers meant by the various terms they used to designate categories of various ranks, I have become a strong advocate of the system recommended in the Code.

The four currently recognized orders of the Myxogastromycetidae: Liceales, Trichiales, Stemonitales and Physarales are reasonably satisfactory except for one genus, *Echinostelium*. Like *Ceratiomyxa, Echinostelium* is the sole representative of its family and includes one extremely common and one or two rare species. It is usually placed in the Stemonitales, where it can be inserted only by indicating its marked differences from all other members of that order. Despite the few species, it seems clear that an Order Echinosteliales must be established to place it where it belongs. This is true, even on the characters of the fructification alone. In addition, recent studies by Alexopoulos (**1960**) have shown that the plasmodium is quite different from that of any other species heretofore cultured, and while we are not yet in position to depend very much on plasmodial characters in keys, I believe they may become of great importance as a morphological basis for taxonomic interpretation.

On the basis of the considerations here advanced, I propose that the Myxomycetes be classified as follows:

Division MYCOTA (FUNGI)
Subdivision MYXOMYCOTINA
Class MYXOMYCETES
Subclass CERATIOMYXOMYCETIDAE
Order *Ceratiomyxales*
Subclass MYXOGASTROMYCETIDAE
Order *Liceales*
Order *Trichiales*
Order *Echinosteliales*
Order *Stemonitales*
Order *Physarales*

If later work should demonstrate that the Plasmodiophorales are more closely allied to the Myxomycetes than I am at present inclined to believe, then a third subclass, the Plasmodiophoromycetidae, will have to be recognized to include that order.

This classification seems to me to be a fairly realistic adjustment, within the hierarchical form of our present system, to our current knowledge of the Myxomycetes. As new information accumulates, it will

undoubtedly have to be altered or discarded, but that is the fate of all attempts at classification. The most I can hope for it is that it will serve for a time.

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LITERATURE CITED

Alexopoulos, Const. J. 1960. Morphology and laboratory cultivation of *Echinostelium minutum*. Am. Jour. Bot. **47**: 37–43.

Bary, A. de. 1858. Ueber die Myxomyceten. Bot. Zeitung **16**: 357–358; 361–364; 365–369.

——. 1859. Die Mycetozoen. Ein Beitrag zur Kenntnis der niedersten Thiere. Zeitschr. Wiss. Zool. **10**: 88–175.

——. 1862. Die neueren Arbeiten über die Schleimpilze und ihre Stellung im System. Flora **45**: 264–272; 282–287; 301–304.

——. 1864. Die Mycetozoen (Schleimpilze). Ein Beitrag zur Kenntnis der niedersten Organismen. W. Engelmann, Leipzig.

Bold, H. C. 1957. Morphology of plants. Harper. New York.

Bonner, John Tyler. 1959. The cellular slime molds. Princeton Univ. Press, Princeton.

Cohen, A. L. 1939. Nutrition of the Myxomycetes. I. Pure culture and two-membered culture of myxomycete plasmodia. Bot. Gaz. **101**: 243–275.

Copeland, Herbert F. 1956. The classification of lower organisms. Pacific Books. Palo Alto.

Cornu, M. 1872. Affinité des Myxomycètes et des Chytridinées. Bull. Soc. Bot. France **19**: 70–71.

Couch, J. N. 1945. Revision of the genus *Coelomomyces*, parasitic in insect larvae. Jour. Elisha Mitchell Soc. **61**: 124–136.

Daniel, J. W., and H. P. Rusch. 1956. Growth of a plasmodial slime mold in pure culture on a soluble medium. Federation Proc. Am. Soc. Exper. Biol. **15**: 513.

Elliott, E. W. 1949. The swarm-cells of Myxomycetes. Mycologia **41**: 141–170.

Famintzen, A., and M. Woronin. 1873. Ueber zwei neue Formen von Schleimpilze: *Ceratium hydnoides* Alb. et Schw. und *Ceratium porioides* Alb. et Schw. Mem. Acad. Imp. Sci. St. Petérsbourg VII. **20**: 1–16.

Fries, E. M. 1829. Systema mycologicum. Myxogastres. **3**: 67–199. E. Mauritius. Gryphiswald.

Gilbert, H. C. 1935. Critical events in the life history of *Ceratiomyxa*. Am. Jour. Bot. **22**: 52–74.

Hagelstein, Robert. 1944. The Mycetozoa of North America. Mineola, N. Y.

Jahn, E. 1908. Myxomycetenstudien 7. *Ceratiomyxa*. Ber. Deutsch. Bot. Ges. **26**: 342–352.

——. 1928. Myxomycetes. In Engler and Prantl, Die natürlichen Pflanzenfam. ed. 2. **2**: 304–339.

Lankester, E. Ray. 1885. Protozoa. In Encycl. Britannica, ed. 9. **19**: 852–891.

Ledingham, G. A. 1934. Zoospore ciliation in the Plasmodiophorales. Nature **133**: 534.

Link, J. H. F. 1833. Handbuch zur Erkennung der nutzbarsten und am häufigsten vorkommenden Gewächse **3**: 405–424. S. J. Joseephy. Berlin.

Lister, Arthur. 1894. A monograph of the Mycetozoa. Ed. 2, revised by G. Lister. 1911. Ed. 3. 1925. British Museum (Nat. Hist.). London.

Macbride, T. H. 1899. The North American slime-moulds. Ed. 2. 1922. Macmillan. New York.

—— **and G. W. Martin.** 1934. The Myxomycetes. Macmillan. New York.

Martin, G. W. 1932. Systematic position of the slime molds and its bearing on the classification of the fungi. Bot. Gaz. **93**: 421–435.

——. 1940. The Myxomycetes. Bot. Review **6**: 356–388.

——. 1949. Myxomycetes. In North Am. Fl. **1** (1): 1–151.

——. 1955. Are Fungi plants? Mycologia **57**: 779–792.

Massee, George. 1892. A monograph of the Myxogasteres. Methuen. London.

Moore, A. R. 1933. On the cytoplasmic framework of the plasmodium, *Physarum polycephalum*. Tohoku Imp. Univ. Sci. Rep. IV. **8**: 189–192.

Moreau, F. 1954. Les champignons, vol. 2. P. Lechevalier. Paris.

Rostafinski, J. T. 1873. Versuch eines Systems der Mycetozoen. Friedr. Wolff. Strassburg.

——. 1874–'76. Śluzowce (Mycetozoa). Monografia. Pamiętnik Towarzystwa Nauk Ścislych w Paryżu **5**: 1–215. 1874; **6**: 218–432. 1875; **8**: 1–45. 1876.

Schinz, Hans. 1920. Myxogastres (Myxomycetes, Mycetozoa). In Rabenhorst, Krypt.-Fl. Deutschlands etc. ed. 2. Akadem. Verlagsgesellschaft. Leipzig.

Schroeter, J. 1885–'86. Die Pilze Schlesiens. In Cohn Krypt.-Fl. Schles. **3** (1): 91–135. J. U. Kern's Verlag. Breslau.

——. 1889. Myxomycetes (Mycetozoa, Schleimpilze, Pilztiere). In Engler and Prantl, Die natürlichen Pflanzenfam. **1** (1): 1–41. W. Engelmann. Leipzig.

Sparrow, F. K. 1959. Interrelationships and phylogeny of the aquatic Phycomycetes. Mycologia **50**: 797–813.

Tippo, O. 1942. A modern classification of the plant kingdom. Chron. Bot. **7** 203–206.

Zopf, W. 1885. Die Pilzthiere oder Schleimpilze. E. Trewendt. Breslau.

NOTES ON BOLETES. XII.

ESTHER A. DICK

The following report concerns observations and studies made in the summers of 1958 and 1959. Those of 1959 were accomplished with the aid of National Science Foundation Grant G 9043.

BOLETUS FIRMUS

Charles Christopher Frost, the "shoemaker botanist" of Brattleboro, Vermont, who did not stick to his last, by the simple procedures of early botany in a rather restricted locality over 75 years ago, turned up a surprising number of new boleti that even the redoubtable Charles Horton Peck with broader opportunities of time and area had not found. Several of Frost's species like *B. pallidus* and *B. griseus* are very common in oak woods over eastern North America at least; some like *B. miniato-olivaceus, B. Russellii, B. speciosus, B. Peckii* and *Leccinum chromapes* (Frost) Singer are not uncommon; the epithet *robustus* had been previously used by Fries and the species is now *Tylopilus eximius* (Peck) Singer; and others are still puzzles. For example, no one is yet quite sure what, with others, *B. sordidus, B. limatulus, B. serotinus* and *B. unicolor* are.

Frost's luridaceous *B. firmus* also has had an uncertain status. It was originally distinguished by distorted growth of pileus and stipe, very firm consistency, gray color, tubes adnate with pores tinged red, and stipe very finely reticulate. Peck had not seen it up to the time of his treatment (**5**, p. 143), although he found it in 1896 (**6**, p. 108). There are no specimens in The New York Botanical Garden, and Murrill of course made no contribution by including the species in his conglomerate *Suillellus luridus* (**3**, p. 17; **4**, p. 151). With some hesitation, Snell and I identified specimens from Tennessee and Michigan as this species. Krieger, however, collected and recognized this species in Canada and comments upon the gray pileus and very fine, pale yellow reticulation of the stipe (**2**, pp. 265–266).

In any event, Krieger was right and *B. firmus* is a good species. In the summer of 1958, on a roadside cut near oaks and other hardwoods in Connecticut just over the Rhode Island line, I found some young fruit bodies and observed their development until they were mature enough to

be identified without question as *B. firmus*. The young stages were naturally firm and brittle, the surface of the pileus a dark brown, and finely subtomentose or finely subfibrillose, the tubes and stipe a bright yellow and the whole rapidly turning bright blue where wounded. The mature carpophores were normal in form and consistency—not at all distorted or irregular, and firm and hard enough but not exceptionally so—although the pileus was rimose-areolate and somewhat fissured and the stipe likewise more or less cracked above and fissured at the base because of the dry atmospheric conditions. The surface of the pileus was matted-tomentose or coactate and subglabrous, subfibrillose or subtomentose only in small areas at the margin, and in color light to dark yellowish-brown, with the cracks pale creamy and the marginal fissures in places a bright orange-yellow. The tubes were a duller yellow, and the pores likewise in part, with the remaining areas brown with a slight reddish tinge but not in any sense red or orange. The stipe was a bright yellow at the apex, a duller yellow downward, yellowish-brown below, with scattered patchy areas of brown and the fissure at the base showing bright reddish-orange, glabrous and finely pale-yellow-reticulate for 1–2 cm. The flesh of the pileus was yellow when fresh, paler to creamy-buff after collection, and that of the stipe a duller yellow with some dark carmine-red at the base and in the worm-holes. The entire inside of fresh specimens turned a bright blue rather rapidly when cut, but a few hours after collection only the lower half of the pileic flesh did so.

The spore-print, which had been recorded by Snell and myself in our tentative description of the species as "probably olivaceous," turned out to be just that. The spores are subelliptical, with only a slight suprahilar depression, very pale colored if at all so, and measure 10–11 or 12 × 3.5–4 μ, about as found in Frost's collections, some of which show some variability in the direction of a slightly greater thickness.

This species then is distinguished among the Luridi with discolorous pores by its dingy grayish-brown or yellowish-brown pileus without tinge of red, orange or olive, its stipe mostly yellow or yellow above and dingy brown below with red only at the base if at all and then only a tinge, the reticulation fine, at first yellow and later brown and extending over not more than the upper third, and the spores not over 15 μ long and mostly under 13 μ.

BOLETUS SUBCLAVATOSPORUS—B. INEDULIS?

Snell described *Boletus subclavatosporus* (Section Calopodes) from North Carolina with Kauffman's specimens and descriptions at hand.

A little later, Murrill described *B. inedulis* from South Carolina to northern Florida. Singer (**7**, p. 34) notes that there are certain discrepancies in the descriptions of these two species such as the slightly different colors of pileus and context, the shape and reticulation of stipe, and irregularity of the shape of the spores, but he feels that these differences are entirely within the range of variation possible or expected in *B. inedulis* (and also for the other species, it might be added). Singer states that he is almost sure that the two are the same and we are inclined to agree, especially, as Singer suggests, until fresh specimens of *B. subclavatosporus* are available.

Under oaks in Swansea, Mass., in 1958, I collected some specimens that were strange to us in this region and which would key out only to the *B. subclavatosporus-inedulis* complex. The differences were of the same sort and in the same direction mentioned in the preceding paragraph, variously from one species to the other. For example, the Massachusetts collection had yellow flesh and tubes as in *B. inedulis* instead of both dingy white as in *B. subclavatosporous,* flesh of stipe dingy white or pale brownish and not changing to blue as in *B. subclavatosporus* instead of yellow and changing to blue as in *B. inedulis.*

What has been considered the main difference, however, is in the spores. Those of *B. subclavatosporus* are clavate, subclavate, diamond-shaped or subcylindric. Those of *B. inedulis* are given by Singer as ellipsoid-fusiform, with some narrowed above (in other words, more or less obclavate). The spores of my Massachusetts collection are, however, not only ellipsoid but, instead of being roughly 8–15 × 3–5 μ as in the other two species, are small—5–10 × 3–4 μ, with the great majority 7–9 × 3.5–4 μ.

In view of all the foregoing, and especially the variability of the spores in the two described species, the best that can be done with this recent collection in the north seems to be to consider it as a small-spored variation of the southern complex, again, to use a perhaps overworked but still necessary expression, until more material of two of the entities is available.

TYLOPILUS BALLOUII

This strikingly orange or reddish-orange species has been known from Long Island, New Jersey, and eastern Pennsylvania southward at least to North Carolina, Tennessee, and Alabama. It is supposed to be rather rare but it is my recollection that Dr. Walker Arde of Philadelphia has sometimes found it in some abundance in New Jersey. In any

event, to my knowledge it has never been collected in the New England States.

It was of great interest, therefore, to receive a cluster of three beautifully colored specimens in a fresh condition collected by Mrs. John G. Curtis near Adamsville, Rhode Island, in September, 1958. They were found in a grassy space in a woodland consisting of oak, beech, maple, tupelo, holly and a very few white pines.

Singer placed *T. Ballouii* in his section Oxydabiles, characterized by "the context changing color when injured (sometimes rather slowly), becoming reddish or grayish violaceous, distinctly reacting with KOH in the species tested" (**7**, p. 90). When the above-mentioned specimens were cut, the flesh of the pileus and stipe changed very slowly to a faint grayish-violaceous or grayish-lilaceous tint. The following reactions of the context in a fresh condition to common chemicals were obtained: KOH—the orange surface and the flesh of the pileus and stipe at once a very bright yellow, the tubes and pores a darker color with a suggestion of a pale dull reddish; $FeSO_4$—the orange surface brownish to blackish, the blacker with time, flesh and tubes both soon bluish-gray; NH_4OH—no effect anywhere; H_2SO_4—surface a very bright red, no effect elsewhere.

A spore-print sufficient in amount for the determination of color was not obtainable. The spores measured in the range 5–9.5 × 3–5 μ, with very few 5 × 3 μ or as broad as 5 μ, with most of them about 8 × 3.7–4 μ.

BOLETUS PALLIDUS

When Singer's Boletineae of Florida first appeared, one of the surprises was to find *B. pallidus* Frost in the Section Calopodes (**7**, p. 31). The Calopodes are characterized especially by having the stipe reticulate or rarely entirely even, and the taste of the flesh more or less bitterish to strongly bitter. Up to the time of Singer's publication, I had never been familiar with these characters in this species in the northern states. Singer did, however (**7**, p. 32), help in tempering my surprise by stating that the stipe is "indistinctly and very faintly reticulate, or with a crude and incomplete network at the very apex, or most frequently perfectly smooth to merely subrugulose," with the taste "mild to bitterish," and adding after the description that *B. pallidus* is intermediate between the Edules and Calopodes in various characters but to his mind better placed with the Calopodes.

Singer's arrangement and conclusions from his Florida studies were accepted, however, especially as in collections in recent years in the

northeast an occasional suggestion both of a bitterness of taste and of reticulation on the upper portion of the stipe was observed. Then in collections made in 1959 in New Hampshire, these two characters were for the first time frequently evident—the taste usually slightly and slowly bitterish but once in a while quite bitter in mature and fresh specimens, and the stipe plainly reticulate for 1 cm at the apex and in a surprising number of cases with a distinct reticulation extending down $\frac{3}{4}$ of the length of the stipe.

BOLETUS MAGNISPORUS

Frost described this rare species briefly and inadequately in 1874 (**1**, p. 103). Peck (**5**, p. 143) repeated this description and added that Morgan found the tomentum of the pileus sometimes brownish-yellow and the flesh greenish-yellow, turning blue when wounded. Frost had not given any flesh characters. Peck and Lloyd never found any specimens as far as can be learned.

Singer (**7**, p. 84) stated that "authentic" material at the Farlow Herbarium showed the spores to be rather small, and *inter alia* commented that they were "rather few and possibly not quite mature," adding that his observations were not sufficient for any conclusions. Snell measured the spores of the Farlow specimens some 20 years ago and these, re-measured recently, were as follows—one specimen, 9.5–14.5 × 3.5–5.5 μ, mostly 13–14 × 5 μ, the other specimens, 9–12 × 3–4.5 μ, mostly 10–11 × 4–4.5 μ. In Frost's type collection at Burlington, Vermont, the spores were found to be large, as Frost and Peck represented them—10–22 × 4.5–9 μ, mostly 14–16 × 5–5.5 μ.

Here then is the situation to date. This species, known only to Frost and possibly Morgan, has been supposed to be distinctive in its *"golden yellow,"* tomentose pileus and rather large spores, with the flesh undescribed but stated by Morgan to be, in what he supposed to be this species, greenish-yellow and turning blue upon exposure, with the stipe yellow above, red below (and presumably glabrous and even), and the spores different in the two extant portions of Frost's collection(s).

In 1959, at Crawford Notch, New Hampshire, I collected two specimens which were entirely unrecognizable by me and offered only two clues—red pores, and spores of size, variable shape and color as precisely matching those of samples from Frost's type of *B. magnisporus* as appeared possible. The pileus was a yellowish-tan and not golden yellow, and tomentose or fibrillose in areas to glabrous otherwise. The flesh was white and did not turn blue. The stipe was yellowish-tan with

a distinct band of red at the very apex, for a distance of about 1 cm from the apex very finely reticulate, with both the reticulation and the even region below more or less mealy in places.

It may appear that with only two suggestive characters, there was little to encourage one to attempt to identify this collection as *B. magnisporus*. Inasmuch, however, as our only available description is incomplete and over 85 years old and covers only the original collection by Frost, the following considerations appear to be reasonable and pertinent. Golden yellow does not always remain so golden, and one may keep in mind Morgan's observation (see **5**, p. 143), for what it is worth, that the tomentum is sometimes brownish-yellow. A tomentum has a way of changing with expansion, splitting, or wearing off. The flesh can be yellow at first and fade with age. The change to blue on bruising or exposure can be very variable, and besides, in this case we have only Morgan's testimony for a specimen which he thought was the same species and for which Frost recorded no change. The red band at the apex is of no definitive importance. And the reticulation at the apex can be very variable in occurrence; see the note on *B. pallidus* above, and compare the situations with respect to *B. subvelutipes* and many other species.

Accordingly, until more is known or more mature ideas have been developed, I am inclined to consider these New Hampshire specimens as the rare *B. magnisporus*.

BOLETUS RUBELLUS SUBSP. FRATERNUS

Another variant specimen from Crawford Notch, New Hampshire, could be no better placed than as *B. rubellus* Krombh. subsp. *fraternus* (Pk.) Sing. The pileus of this specimen, rather desiccated at the time of collection, was mostly olive buff (between Olive Buff and Dark Olive Buff but not at all Ridgway's Deep Olive Buff which is between these two), but where it had been chewed away by some animal, the newly forming surface was a pinkish-red. The flesh was whitish instead of pale to deep yellow; the pores of the tubes were slightly red in places. The stipe was yellowish with some red areas. Otherwise, the characters, including the bluing of the various parts, agreed with those ordinarily given.

Thus far, this subspecies, common in the southern states as far as central Florida (but not in the tropical zone), has been known only as far north as Massachusetts. If the above identification is correct, this collection represents an extension of the range somewhat to the northeastward.

SUILLUS PINORIGIDUS

This species, described originally by Snell and Dick as associated with *Pinus rigida* in Rhode Island and since found on Cape Cod, Massachusetts, on Long Island, and near Schenectady, New York, probably occurs throughout the range of the pitch pine. I found it in quantity in the fall of 1959 at Madison, New Hampshire.

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LITERATURE CITED

1. **Frost, Charles C.** 1874. Catalogue of Boleti of New England, with descriptions of new species. Bull. Buffalo Soc. Nat. Sci. **2**: 100–105.
2. **Krieger, L. C. C.** 1936. The Mushroom Handbook. New York.
3. **Murrill, William A.** 1909. The Boletaceae of North America—I. Mycologia **1**: 4–18.
4. ——. 1910. Agaricales. Family 6. Boletaceae. North American Flora **9**: 133–161.
5. **Peck, Charles H.** 1889. Boleti of the United States. Bull. N. Y. State Mus. **2**: 73–166.
6. ——. 1897. Ann. Rep. State Bot. (for 1896). Ann. Rep. N. Y. State Mus. **50**: 77–159.
7. **Singer, Rolf.** 1947. The Boletoideae of Florida. The Boletineae of Florida with notes on extralimital species III. Amer. Midland Nat. **37**: 1–126.

INTERRELATIONS BETWEEN GROWTH RATE AND NUCLEAR RATIOS IN HETERO-KARYONS OF NEUROSPORA CRASSA

DEANA T. KLEIN [1]

(WITH 3 FIGURES)

The concept that "gene dosage" may be a factor in growth responses in fungi has not received the attention such an important idea merits. Beadle and Coonradt in 1944 proposed that some genes may have a high efficiency of action in heterokaryons of *Neurospora crassa* Shear and Dodge. Nuclei containing such genes could constitute a very small proportion of the total genic complement of heterokaryons and permit normal rates of growth. Beadle and Coonradt (**1944**) constructed a hypothetical efficiency curve for a given gene by plotting the theoretical rate of growth against nuclear proportions and concluded that in a heterokaryon containing two sorts of nuclei each with a different deficient gene, the two curves might intersect. Since no particular one of the many ratios allowing normal rate of growth would be favored, heterokaryons exhibiting normal growth could be expected to have varying ratios of nuclei. They also concluded that heterokaryons possessing nuclei containing inefficient or deficient genes might exhibit submaximal rates of growth and should, therefore, have only one fixed ratio of nuclear types.

Prout et al. (**1953**) agreed that rate of growth might be a function of the ratios of the nuclear components constituting the heterokaryon. They further suggested that those hyphae capable of growing at maximal rates would soon constitute the advancing edge of the mycelium.

Pittenger and Atwood (**1956**) disagreed with this concept because it did not explain the stable, submaximal rates of growth reported by Barratt and Garnjobst (**1949**) in heterokaryons composed of wild type and colonial-1 homokaryons. Pittenger and Atwood (**1956**) found that

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heterokaryotic cultures started with extreme disproportion of input ratios exhibited submaximal growth rates and these rates were stable for the course of the experiment.

Techniques are available to evaluate these conflicting points of view. It has been determined that there is random distribution of nuclei in the macroconidia of *Neurospora crassa* (Prout et al., **1953**; Klein, **1958**) so that numbers and types of nuclei in these conidia can be subjected to statistical analysis.

Data are presented which bear on the relationships between rates of growth and gene dosage. Growth rate appeared to be a function of nuclear ratio in that a "threshhold effect" was observed. Nuclear ratios below a certain level did not allow normal rates of growth.

MATERIALS AND METHODS

Hyphal tip isolates from young heterokaryons were used (Klein, **1958**) in preference to heterokaryons prepared by the method of Pittenger and Atwood (**1956**) in which masses of conidia were used. The technique described here is simpler than that used by Pittenger and Atwood (**1956**), the heterokaryotic mycelium is "forced" immediately since the parents are auxotrophic, and only minimal medium is used. Thus, whatever selective forces may operate to determine nuclear ratios may proceed more rapidly when growth is confined to unsupplemented medium.

The homokaryotic, macroconidial stocks of *Neurospora crassa* used were lysineless (4545A); pantothenicless (5531A); and p-aminobenzoicless (1633A). Fries' synthetic medium was supplemented with the appropriate growth factors when required (Ryan, **1950**). A concentration of 40 μg/ml lysine, 2 μg/ml calcium pantothenate, or 5 μg/ml p-aminobenzoic acid each elicited a maximal growth rate response of the homokaryotic mutant. All cultures were incubated at 25° C.

Heterokaryons were established in the following manner. The homokaryotic mutant strains were grown on the appropriately supplemented agar in 50 ml Erlenmeyer flasks for 7 days. Conidia were washed off the surface of the agar with 30 ml sterile distilled water and filtered 3 times through sterile glass wool to remove hyphal fragments. The densities of the 2 suspensions were adjusted to equal turbidity using a Klett colorimeter fitted with an amber filter. Aliquot portions of the 2 suspensions were combined in various proportions. The numbers of viable conidia, which determined the actual input ratio, were obtained by plating the original suspensions of homokaryotic conidia, appropriately di-

luted, on minimal medium containing 0.75% sorbose, 0.1% sucrose, and the appropriate growth factors. The mixtures of conidia were centrifuged, the supernatant decanted, and a loopful of conidial pellet placed centrally on minimal agar in a Petri dish. After 24–48 hours at 25° C, hyphal tips were isolated from the periphery of the colony and placed on minimal agar in test tubes. These cultures will be referred to as the original stock tubes.

Growth rates and nuclear ratios were determined for these hyphal tip heterokaryons in the following way. If growth occurred in the original stock tubes, 2 sister cultures were started for each hyphal tip heterokaryon by inoculating conidia into a growth tube ca. 300 mm long containing 10 ml minimal medium (Ryan, **1950**) and into a 50 ml flask containing 10 ml minimal medium. Rate of growth was determined by marking the growth tube at suitable intervals of time to indicate the advance of the frontier of the mycelium. After 7 days growth in the flask, conidia were washed off with sterile distilled water, filtered 3 times through glass wool, diluted appropriately, and plated in minimal agar medium containing 0.75% sorbose, 0.1% sucrose, and the appropriate growth factors. A replication of 6 plates per medium was used and the colonies were counted after 3 days.

In a few early experiments hyphal tips were inoculated directly into growth tubes. When the advancing edge reached the end of the tube (2 to 7 days growth) the conidia were washed off the surface of the agar, filtered through glass wool, diluted appropriately and plated as described above. The results obtained with respect to the relationship between growth rate and nuclear ratios were completely in accord with the results obtained by plating conidia from the sister culture grown in a small flask instead of the conidia from the growth tube. For 2 reasons it was felt to be justified to use the sister cultures for routine plating: 1) Possible differences due to differences in age of conidia plated would be eliminated; 2) use of conidia from the growth tube for plating would preclude saving the original heterokaryons.

The average number of nuclei per conidium was determined by counting the nuclei in ca. 500 conidia, using in each case the same heterokaryotic conidial suspension as was plated. The conidia were stained employing a modification (Klein, **1958**) of the technique described by Huebschman (**1952**). The proportion of lysineless nuclei in each hyphal tip heterokaryon was estimated by the "approximate method" of Atwood and Mukai (**1955**). The "approximate method" has been shown to give results which agreed very closely with those obtained using the "iterative method" of Prout et al. (**1953**) (Klein, **1958**).

EXPERIMENTAL RESULTS

Heterokaryon A: lysineless + pantothenicless: All cultures of viable hyphal tips were scored for rates of growth and a large percentage of these were assayed to determine the proportions of lysineless nuclei.[2] TABLE I summarized some of these data for heterokaryon A. The average rate of growth for each experiment was determined using those isolates started with an estimated input of 50% lysineless conidia. It was felt that a control rate of growth could best be determined by using the "50% input" heterokaryons because equal numbers of 2 types of

TABLE I

RATES OF GROWTH AND PROPORTIONS OF LYSINELESS NUCLEI IN REPRESENTATIVE HETEROKARYOTIC ISOLATES CONSTITUTED WITH LYSINELESS + PANTOTHENICLESS NUCLEI (HETEROKARYON A)

Experiment No.	% lysineless conidia		Original culture		
	Estimated input	Actual input	Growth rate		p ≈ *
			mm/hr	% control†	
4	1.0	1.3	3.4	87.0	0.03
4	1.0	1.3	3.5	89.8	0.19
4	1.0	1.3	3.7	95.2	0.25
4	1.0	1.3	3.9	100.0	0.16
4	10.0	12.7	3.8	97.2	0.39
4	10.0	12.7	3.8	97.2	0.22
4	10.0	12.7	4.4	112.9	0.32
4	10.0	12.7	4.4	112.9	0.07
5	50.0	37.5	4.0	97.0	0.04
5	50.0	37.5	1.7	41.0	0.05
6	50.0	33.7	3.5	100.8	0.11
6	50.0	33.7	3.5	100.8	0.10
5	90.0	85.7	4.2	101.5	0.14
5	90.0	85.7	4.0	97.0	0.40
6	90.0	83.6	0.9	26.5	0.04
6	90.0	83.6	3.5	100.8	0.32
5	99.0	98.4	1.2	28.9	0.07
5	99.0	98.4	0.6	14.5	0.001
5	99.0	98.4	4.2	101.5	0.11
5	99.0	98.4	0.9	21.7	0.04
6	99.0	98.0	0.9	25.9	0.02
6	99.0	98.0	3.5	100.9	0.18
6	99.0	98.0	1.6	46.1	0.05
6	99.0	98.0	1.1	31.7	0.03

* p ≈ is proportion of lysineless nuclei.

† Per cent control determined for each experiment using the average rate of growth of isolates with 50% input (estimated) for that experiment. Average rate for Experiment No. 4 is 3.9; for 5, 4.14; for 6, 3.47.

[2] The symbol p ≈ is used to denote the proportion of lysineless nuclei as determined by the "approximate method" (Atwood and Mukai, 1955).

nuclei would probably allow for maximal random anastomoses and maximal random mixing of the nuclei. The rates of growth of at least 5 isolates were averaged for each experiment, excluding the rare slow-growing isolate. Thus, the rate of growth of each heterokaryotic isolate listed in TABLE I must be compared to the average rate for the particular experiment. Results are given as a percentage of the control in each experiment because the average rates of growth varied between the experiments by as much as 15%.

There was no strict relationship between input of lysineless conidia (determined by viable count) and proportions of lysineless nuclei in the resulting heterokaryons; even a preponderance of lysineless conidia in the original inoculum did not result in hyphal tip cultures with a preponderance of lysineless nuclei. This finding is at variance with that of Pittenger et al. (**1955**) who reported a positive correlation between the input (calculated) and output (determined) percentages when masses of conidia were used to start heterokaryotic growth.

The data here show that $p \approx$ varied between 0.001 and 0.40 and the rates of growth varied between 0.62 and 4.40 mm/hr. There was a positive correlation between submaximal rate of growth (any rate less than ca. 89% of control) and a $p \approx$ of ca. 0.07 or less; in only 2 instances was a very low $p \approx$ found (0.04 and 0.07) when the isolates grew at maximal rates. Growth rate was maximal over a wide range of $p \approx$'s. This finding agrees with the hypothesis suggested by Beadle and Coonradt (**1944**). In contrast to their hypothesis, however, those isolates exhibiting submaximal rates of growth did not have one fixed ratio, but also exhibited a wide range of $p \approx$'s. Examination of the variations in $p \approx$ exhibited by isolates showing submaximal rates of growth and the variations in $p \approx$ exhibited by isolates showing maximal rates of growth indicates that the range of values is as great in slow-growing as in rapidly-growing cultures.

FIG. 1 illustrates the type of growth responses of a few representative A heterokaryons. It should be noted that the rates of growth were constant during the course of the experiment.

Heterokaryon B: lysineless + p-aminobenzoicless: The isolates of heterokaryon B showed responses similar to heterokaryon A but differed in detail (TABLE II). The average rate of growth for each experiment was determined in the same way as it was for heterokaryon A; the rate of growth of each heterokaryotic isolate listed in TABLE II must be compared to the average rate of the proper experiment. Again, there was no correlation between input of lysineless conidia and the proportion of lysineless nuclei in the hyphal tip cultures. The $p \approx$ varied between

0.12 and 0.81 and the rates of growth varied between 1.92 and 4.10 mm/hr. No isolate of B exhibited as slow a rate of growth as any of the A heterokaryons. There was no equivalence between absolute rate of growth and $p \approx$ except that cultures growing at less than ca. 89% of the control rate of growth had a $p \approx$ of ca. 0.31 or less. A single exception was found in which an isolate with a low $p \approx$ (0.25) grew at a maximal rate. Growth rate was maximal over a wide range of $p \approx$'s. Isolates growing at submaximal rates also showed a wide range of $p \approx$'s. FIG. 2 illustrates rates of growth of representative B heterokaryons.

Effect of supplementation on rate of growth: The slow rates of

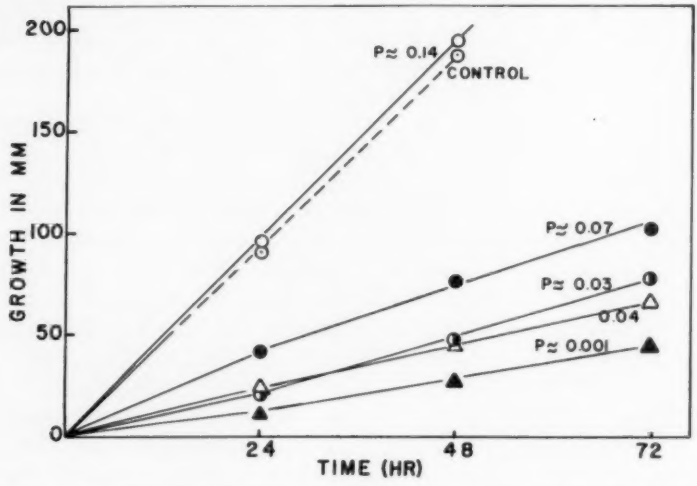

FIG. 1. Linear growth curves of representative isolates of heterokaryon A (lysineless + pantothenicless). The proportion of lysineless nuclei ($p \approx$) is shown for each isolate.

growth could be reproduced so long as conidia in the original stock tube were available. Thus it was possible to save these tubes for some time and be assured of a supply of conidia which would give rise to a slow-growing mycelium. It was possible to test the effects on rate of growth of supplementation of the medium.

The growth rate of an isolate of heterokaryon A already growing at a maximal rate was unaffected by the presence of calcium pantothenate or lysine in the minimal medium. The growth rate of an isolate grow-

ing at a submaximal rate was unaffected by the addition of lysine at 4 μg or 40 μg/ml or the addition of 0.02 μg/ml calcium pantothenate (FIG. 3, A). Rate of growth of the pantothenicless homokaryon is shown in FIG. 3, B. The addition of 0.2 μg/ml pantothenate, however, allowed the slow-growing heterokaryon to grow immediately at a maximal rate. If conidia from the end of the growth tube containing 0.2 μg/ml pantothenate were transferred to minimal medium, the rate of growth returned to that of the original culture. This experiment

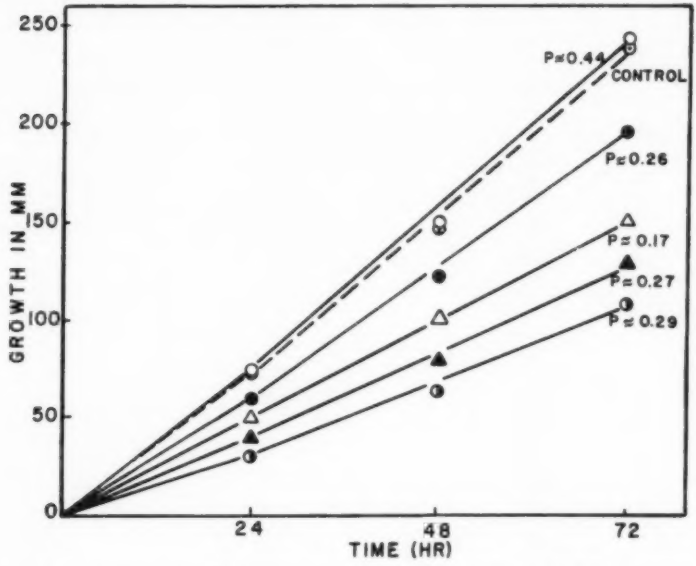

FIG. 2. Linear growth curves of representative isolates of heterokaryon B (lysineless + p-aminobenzoicless). The proportion of lysineless nuclei (p ≈) is given for each isolate.

was repeated with similar results using another slow-growing isolate. Pittenger and Atwood (1956) have described essentially the same results for their heterokaryons.

DISCUSSION

Hyphal tips were isolated from two types of heterokaryons, each having the lysineless nucleus in common. The conidial progeny of viable hyphal tips were examined to determine rates of growth and pro-

portions of lysineless nuclei. Those cultures growing at maximal rates exhibited a wide range of proportions of lysineless nuclei. The slow-growing isolates had no one fixed ratio of one mutant type to the other, as Beadle and Coonradt (**1944**) proposed, but there was a particular proportion of lysineless nuclei below which the heterokaryons grew at submaximal rates. This proportion varied with the mutant combination; for the lysineless + pantothenicless heterokaryons the p ≈ was ca. 0.07

TABLE II

RATES OF GROWTH AND PROPORTIONS OF LYSINELESS NUCLEI IN REPRESENTATIVE HETEROKARYOTIC ISOLATES CONSTITUTED WITH LYSINELESS + P-AMINOBENZOICLESS NUCLEI (HETEROKARYON B)

Experiment No.	% lysineless conidia		Original culture		
	Estimated input	Actual input	Growth rate		p ≈ *
			mm/hr	% control†	
3	0.5	0.3	4.1	107.9	0.67
3	0.5	0.3	3.9	102.7	0.57
3	0.5	0.3	2.1	55.2	0.16
3	0.5	0.3	3.1	81.6	0.27
2	1.0	0.75	3.2	84.3	0.23
2	1.0	0.75	3.2	84.3	0.20
1	50.0	25.3	2.5	69.0	0.12
1	50.0	25.3	3.5	97.8	0.34
3	50.0	42.2	3.8	100.0	0.25
3	50.0	42.2	4.0	105.5	0.44
3	50.0	42.2	3.4	89.6	0.36
1	99.0	97.2	3.9	107.8	0.65
1	99.0	97.2	3.9	107.8	0.33
2	99.0	98.7	3.7	97.4	0.81
2	99.0	98.7	3.2	84.3	0.31
3	99.5	99.4	2.1	55.3	0.16
3	99.5	99.4	3.9	102.7	0.40
3	99.5	99.4	3.9	102.7	0.49
3	99.5	99.4	2.0	52.6	0.26
3	99.5	99.4	1.9	50.0	0.29

* p ≈ is proportion of lysineless nuclei.

† Per cent control determined for each experiment using the average rate of growth of isolates with 50% input (estimated) for that experiment. Average rate for Experiment No. 1 is 3.62; for 2, 3.8; for 3, 3.8.

or less while for the lysineless + p-aminobenzoicless heterokaryons it was 0.31 or less.

Each individual hyphal tip heterokaryon contained a specific proportion of lysineless nuclei which was usually unrelated to the input ratio. It may be that certain nuclear ratios did not allow for continued growth of the individual hypha, in which case, these particular hyphae were never sampled.

The effect on the growth rate of certain slow-growing lysineless + pantothenicless heterokaryons by supplementation of the minimal medium with lysine or calcium pantothenate gave results which might be expected from their nuclear constitution. One heterokaryon contained 3% lys⁻pan⁺ nuclei ($p \approx = 0.03$) and 97% lys⁺pan⁻ nuclei. In view of the low percentage of nuclei that were pan⁺, there was most probably a deficiency in the endogenous amount of pantothenate available for participation in enzymatic reactions, such deficiency resulting in a decreased rate of growth. The slow-growing heterokaryon, having insufficient pantothenate, would grow at a maximal rate when supplied

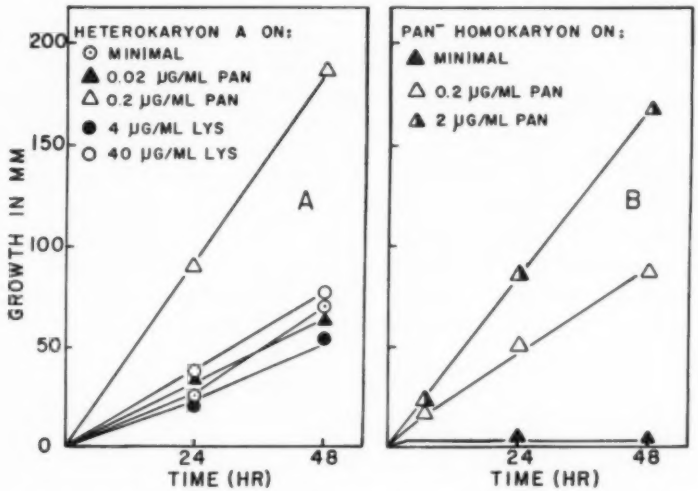

FIG. 3. A. Effect of supplementation of minimal medium with lysine or calcium pantothenate on linear growth of a slow-growing isolate of heterokaryon A with an initial nuclear proportion of 0.03 lys⁻pan⁺ nuclei and 0.97 lys⁺pan⁻ nuclei. B. Linear growth of the pantothenicless homokaryon (5531A) on several concentrations of calcium pantothenate.

with as little as 0.2 µg/ml pantothenate. The growth of the pantothenicless homokaryon on medium containing 0.2 µg/ml calcium pantothenate was shown in FIG. 3, B and found to be about 45% of the rate shown on 2.0 µg/ml. The rates of growth of the slow-growing heterokaryon on minimal medium and that of the pantothenicless homokaryon on medium containing 0.2 µg/ml pantothenate were about the same. Were the conclusions of Pittenger and Atwood (**1956**) correct, an exogenous

level of 0.2 μg/ml of pantothenate should have the same growth-promoting effect as 3% pan$^+$ nuclei ($p \approx = 0.03$). However, slow-growing heterokaryons may have growth rates similar to the above isolate but have a $p \approx$ varying between 0.02 and 0.07. Thus, there may not be a direct relationship between nuclear proportions and growth factor synthesis.

Pittenger and Atwood (**1956**) had shown that curves of a) growth rate plotted against the proportion of pan$^+$ nuclei (lys$^-$) in individual heterokaryons and b) growth rate plotted against concentration of pantothenate for the pan, al-1 homokaryon were superimposable. They concluded that the proportion of pan$^+$ nuclei could be equated to the concentration of pantothenate in the medium; that is, a certain proportion of pan$^+$ nuclei in a particular heterokaryon had the same growth effect as a certain concentration of pantothenate in the medium. The identical graph was made using those pertinent data shown in TABLE I and standard curves for the growth of the pantothenicless homokaryon. There was no such nice correspondence found between proportions of pan$^+$ nuclei and μg pantothenate. Instead the hyphal tip heterokaryons fell into 2 distinct groups: 1) Rates of growth below 2.0 mm/hr: $p \approx$ of 0.07 or less; 2) rates of growth above 2.0 mm/hr: $p \approx$ of above 0.07. The pertinent data of TABLE II were also plotted and again the clustering of $p \approx$'s was seen: 1) Rates of growth below 3.3 mm/hr: $p \approx$ of 0.31 or less; 2) rates of growth above 3.3 mm/hr: $p \approx$ of above 0.31.

It is suggested that the original nuclear proportions in mycelial growth resulting from viable hyphal tips most probably determined the original rates of growth; that is, there was a threshhold effect of nuclear ratios on growth rate in the hyphal tip cultures.

ACKNOWLEDGMENTS

The author wishes to thank Dr. F. J. Ryan in whose laboratory the work was carried on for his suggestions and for many interesting discussions concerning heterokaryosis. Thanks are also extended to Dr. Salome Waelsch for her critical examination of the manuscript.

SUMMARY

The interrelations of growth rate and proportions of lysineless nuclei in heterokaryotic hyphal tip isolates of *Neurospora crassa* were studied. Those isolates growing at maximal and submaximal rates showed a wide range of nuclear ratios. Submaximal rates of growth were cor-

related with certain minimal proportions of lysineless nuclei; that is, there was a minimal or threshhold value for lysineless nuclei, below which the hyphal tip heterokaryons grew at submaximal rates. The minimal values varied with the type of heterokaryon: for lysineless + pantothenicless isolates it was 7% or less; for lysineless + p-aminobenzoicless isolates, 31% or less.

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LITERATURE CITED

Atwood, K. C., and F. Mukai. 1955. Nuclear distribution in conidia of *Neurospora* heterokaryons. Genetics **40**: 438–443.

Barratt, R. W., and L. Garnjobst. 1949. Genetics of a colonial microconidiating mutant strain of *Neurospora crassa*. Genetics **34**: 351–369.

Beadle, G. W., and V. L. Coonradt. 1944. Heterocaryosis in *Neurospora crassa*. Genetics **29**: 291–308.

Huebschman, C. 1952. A method for varying the average number of nuclei in the conidia of *Neurospora crassa*. Mycologia **44**: 599–604.

Klein, D. T. 1958. Randomness of nuclear distribution in conidia of *Neurospora* heterokaryons. Z. Vererbungslehre **89**: 323–327.

Pittenger, T. H., A. W. Kimball, and K. C. Atwood. 1955. Control of nuclear ratios in *Neurospora* heterokaryons. Amer. Jour. Bot. **42**: 954–958.

——, **and K. C. Atwood.** 1956. Stability of nuclear proportions during growth of *Neurospora* heterokaryons. Genetics **41**: 227–241.

Prout, T., C. Huebschman, H. Levene, and F. J. Ryan. 1953. The proportions of nuclear types in *Neurospora* heterokaryons as determined by plating conidia. Genetics **38**: 518–529.

Ryan, F. J. 1950. Selected methods of *Neurospora* genetics. Meth. Med. Res. **3**: 51–75.

THE CULTIVATION OF PATHOGENIC FUNGI ON A MOLYBDENUM MEDIUM [1]

JOHN A. MACLAREN

Molybdenum in the form of phosphomolybdic acid has been shown to cause a distinctive pigmentation of *Candida albicans* (**6**). Furthermore, a culture medium containing phosphomolybdic acid has been suggested as a useful laboratory aid in identifying *C. albicans*, either in pure cultures or in the presence of closely related species (**6**).

In the laboratory identification of pathogenic fungi by culturing methods, a number of organisms may be encountered in the form of yeasts. Besides species of *Candida*, they include not only the yeasts, *Cryptococcus neoformans* and *Torulopsis glabrata*, and the pathogenic species of *Geotrichum* and *Trichosporon*, but also the yeast phase forms of *Sporotrichum Schenckii*, *Blastomyces dermatitidis*, *Blastomyces brasiliensis* and *Histoplasma capsulatum*. This research was undertaken to explore the possibility that one or more of these pathogenic fungi might be confused with *C. albicans* on initial isolation, in which the molybdenum medium was employed. The growth of such organisms on the molybdenum medium was studied in an effort to define the selective and differential properties of the medium more fully.

It is the purpose of this communication to report the growth characteristics of a variety of pathogenic fungi when cultured on the molybdenum medium, and to provide additional evidence for the specific nature of the medium in identifying *C. albicans*.

MATERIALS AND METHODS

The fungi used in this study, with the exception of *C. albicans* and one strain of *C. neoformans*, were obtained from other laboratories through the courtesy of Dr. Libero Ajello (C.D.C.), Dr. John W. Carmichael (J.W.C.), Dr. Chester W. Emmons (C.W.E.), Dr. Margarita Silva (M.S.), Dr. G. A. de Vries of the Centraalbureau voor Schimmelcultures (C.B.S.), and Dr. Lynferd J. Wickerham (N.R.R.L.). The organisms have been described in the works of Carmichael (**2**, **3**), Conant and co-authors (**4**), Lodder and Kreger-Van Rij (**5**), Martin and co-workers (**7**) and Wickerham (**8**).

[1] This work was supported in part by a grant from the Winthrop Laboratories, New York, N. Y.

The time required for the growth of adequate inoculums varied with the different fungi, ranging from 24 hours with *C. albicans* to four or five days in the case of *H. capsulatum*. With the exception of *C. albicans, C. neoformans* and *T. glabrata,* which were cultured on Sabouraud's glucose agar, the organisms were grown on brain heart infusion agar at 36° C prior to the inoculation of the molybdenum medium.

The molybdenum medium was prepared according to the original method (**6**) with the exception that Merck phosphomolybdic acid was employed ($20MoO_3 \cdot 2H_3PO_4 \cdot 48H_2O$). It had previously been established that Merck phosphomolybdic acid gave satisfactory results in differentiating the *Candida* yeasts. To recapitulate, a basal medium was prepared consisting of proteose-peptone (Difco) 1 per cent, sucrose 4 per cent, agar 1.5 per cent and distilled water. After adjusting the pH to 7.6 the medium was autoclaved at 10 pounds pressure for 15 minutes. To 100 ml of the basal medium, cooled to 50–55 C, was added 1.5 ml of a 12.5 per cent aqueous solution of phosphomolybdic acid, to give a final concentration of 1.9 mg/ml. The precipitate formed was suspended by agitation and the medium distributed into Petri dishes.

The medium was inoculated by taking cells directly from a slant of

Table I

Characteristics of streak-form of growth of some pathogenic fungi

Organism	Source	No.	Growth*	Color (reflected light)	
				Surface	Bottom
Sporotrichum Schenckii	C.D.C.	A-874	++	light gray to blue-gray	light gray to blue-gray
Sporotrichum Schenckii	C.W.E.	7034	++	light tan to tan	light tan to tan
Sporotrichum Schenckii	M.S.	1044.3	++	white	light gray to gray
Cryptococcus neoformans	C.W.E.	3749	+	light gray to blue-gray	light gray to dark gray
Cryptococcus neoformans	J.A.M.	C.N.	+++	blue	blue
Cryptococcus neoformans	M.S.	1499:116	++	light gray to blue-gray	light gray to blue-gray
Cryptococcus neoformans	M.S.	1473	+++	blue-gray	dark blue-gray
Geotrichum candidum	J.W.C.	S77, S80, S85, S86	+	light gray	light gray
Geotrichum amycelium	J.W.C.	S104, S106	+++	olive	olive
Trichosporon cutaneum	J.W.C.	S-217	+++	light gray to gray	light gray to gray
Trichosporon cutaneum	C.B.S.	2468	+++	light tan to olive	tan to olive
Trichosporon infestans	C.B.S.	2530	+++	light gray	light gray
Torulopsis glabrata	N.R.R.L.	Y2241, Y396	++	light gray	light gray
Candida albicans	J.A.M.	A	+++	medium to dark olive or dark gray	medium to dark olive or dark gray

* Growth: +++ = Good; ++ = Fair; + = Sparse.

TABLE I—*Continued*

Organism	Extracellular reaction**		Surface	Microscopic examination	Consistency of growth
	Reflected light	Transmitted light			
Sporotrichum Schenckii	–	–	dull and rough	yeast cells	butyrous
Sporotrichum Schenckii	–	–	dull and rough	filaments	membranous
Sporotrichum Schenckii	–	–	dull and rough	filaments	membranous
Cryptococcus neoformans	–	–	smooth, moist, glistening	yeast cells	viscid
Cryptococcus neoformans	±, gray	±, gray	smooth, moist, glistening	yeast cells	viscid
Cryptococcus neoformans	–	–	smooth, moist, glistening	yeast cells	viscid
Cryptococcus neoformans	±, gray	±, gray	smooth, moist glistening	yeast cells	viscid
Geotrichum candidum	—	—	dull and rough	mainly filaments, and a few yeast cells	usually membranous, sometimes butyrous
Geotrichum amycelium	–	–	dull and rough	mainly filaments, and a few yeast cells	usually membranous, somtimes butyrous
Trichosporon cutaneum	–	–	dull and rough	mainly filaments, some yeast cells	butyrous
Trichosporon cutaneum	—	—	dull and rough	filaments	membranous
Trichosporon infestans	–	–	dull and rough	filaments	membranous
Torulopsis glabrata	—	—	smooth and glistening	yeast cells	butyrous
Candida albicans	–	–	smooth and glistening	yeast cells	butyrous

** Extracellular reaction: ± = Weak; – = No reaction.

the appropriate organism and streaking them in a wide zone across the agar surface. Following incubation of the cultures at 36° C for 48 hours, the results were recorded.

RESULTS

Yeast phase cultures of *H. capsulatum* (three strains) and *B. dermatitidis* (three strains) grew very poorly and those colonies which did appear were rough and generally light gray to gray in color. *B. brasiliensis* (one strain) failed to grow on the medium. Considering the growth characteristics of these organisms, it was clear that none of them resembled *C. albicans*.

The characteristics of those fungi which achieved better growth than the yeast phase organisms mentioned above, are recorded in TABLE I. While the yeast phase cultures of *S. Schenckii* grew more profusely than the other yeast phase fungi, they were distinctly different from *C. albicans* by virtue of their color and form. It was anticipated that the

selective and differential properties of the molybdenum medium would be put to a thorough test in distinguishing *C. albicans* from the more closely related pathogenic species of *Cryptococcus, Torulopsis, Geotrichum* and *Trichosporon*. It was apparent that *C. albicans* could be clearly distinguished from these organisms on the basis of pigmentation and morphology.

Of all the organisms included in the study, only two strains of *C. neoformans* gave rise to an extracellular reaction, and at 48 hours of incubation the reaction was weak. The infrequency of the extracellular reaction is striking in view of its occurrence among *Candida* yeasts, in which three of seven species were found to possess the property (**6**).

To reveal the possible toxicity of the phosphomolybdic acid in the medium, an experiment was performed to ascertain the inhibitory effect of the molybdenum compound upon the growth of the test organisms. The basal medium, without the phosphomolybdic acid and having the same final pH of 5.3, was used to grow the fungi under cultural conditions similar to those previously described. The results obtained were essentially the same as those observed using the molybdenum medium. *B. brasiliensis* did not grow, and the remaining organisms exhibited the same degree of growth as in the presence of phosphomolybdic acid. It was clear that under the experimental conditions, the phosphomolybdic acid did not inhibit the growth of the fungi.

DISCUSSION

Considering the varied growth requirements of the fungi studied (**1**) it is not surprising that on the molybdenum medium some of them grew poorly and one, not at all. Undoubtedly these results, as exemplified by *H. capsulatum, B. dermatitidis* and *B. brasiliensis*, can be attributed to at least one or more of the following characteristics of the medium, acidic pH (5.3), low concentration of growth factors and sucrose as the source of carbohydrate.

That molybdenum was not toxic for these fungi was borne out by the experiment employing the basal medium alone. The presence or absence of phosphomolybdate in the medium was without observable effect on the growth of the organisms.

The selective growth property of the medium is, therefore, not due to the presence of the molybdenum compound, but rather to the nutritional characteristics of the medium. It is also the nutritional constituents of the medium which have been shown to be very important for its differential property (**6**). These properties of the medium were

so specific, that, while some of the test organisms became markedly pigmented, such as *C. neoformans* and *Geotrichum amycelium*, none of the fungi could be confused with *C. albicans*, whose growth characteristics were highly distinctive.

SUMMARY

1. None of the pathogenic fungi cultured on the molybdenum medium could be confused with *C. albicans*.

2. The selective and differential properties of the medium make it highly specific for the identification of *C. albicans*.

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LITERATURE CITED

1. **Area Leao, A. E. and A. Cury.** 1950. Deficiencias vitaminicas de cogumelos patogenicos. Mycopath. et Mycol. Appl. **5**: 65–90.
2. **Carmichael, J. W.** 1954. A taxonomic study of the genus *Geotrichum*. Harvard University, Ph.D. Thesis. 207 pp.
3. **Carmichael, J. W.** 1957. *Geotrichum candidum*. Mycologia **49**: 820–830.
4. **Conant, N. F., D. T. Smith, R. D. Baker, J. L. Callaway and D. S. Martin.** 1954. Manual of clinical mycology. Ed. 2, W. B. Saunders Co., Philadelphia. 456 pp.
5. **Lodder, J. and N. J. W. Kreger-Van Rij.** 1952. The Yeasts—A Taxonomic Study. Interscience Publishers, Inc., New York. 713 pp.
6. **MacLaren, J. A. and D. Armen.** 1958. Pigmentation of *Candida albicans* by molybdenum. Amer. J. Clin. Path. **30**: 411–422.
7. **Martin, D. S., C. P. Jones, K. F. Yao and L. E. Lee, Jr.** 1937. A practical classification of the monilias. Jour. Bact. **34**: 99–129.
8. **Wickerham, L. J.** 1957. Apparent increase in frequency of infections involving *Torulopsis glabrata*. J. A. M. A. **165**: 47–48.

NOTES AND BRIEF ARTICLES

A Large Collection of a Rare Fungus

The fungus *Fistulina brasiliensis* O. & K. Fidalgo (**1958**) was found only once in the type locality (Tijuca Forest, Rio de Janeiro, Brazil, alt 300 m) since its discovery in March 1957.

Last summer, in São Paulo city (400 km WSW of Rio de Janeiro, alt 800 m), during an exceptionally good season for collecting higher hymenomycetes, a large collection of *F. brasiliensis* was found in the woods of the São Paulo Botanical Garden (Instituto de Botânica). The first carpophore, a small, juvenile fruiting body, was found in January. Two weeks later more than fifty specimens were found in the same area. Many were carefully followed through the ground until reaching the roots to which they were attached. All the roots originated from a dead trunk of *Piptadenia colubrina* Benth., commonly known in São Paulo state as "angico branco," and elsewhere as "cambuí-angico." This may indicate that the fungus is corticicolous, acting as a parasite on the root system of that leguminous tree.

This large collection enabled us to better understand the variability of the morphology of the carpophore which is here redescribed:

Carpophore always stipitate. Stipe lateral or falsely excentric, 0.5–2.3 cm in diam., 1–15.5 cm long. The length of the stipe depends in part on how deep is the root to which it is attached.

Fig. 1. Fruiting bodies of *Fistulina brasiliensis* found on roots of *Piptadenia colubrina*.

Pileus frequently lobate, 0.5–11 × 1–13 cm, 0.8–10 mm thick, margin smooth, undulate or with small indentations.

Context 0.4–7 mm thick, thicker towards the point of attachment to the stipe.

Tubes 0.4–3 mm long.—OSWALDO FIDALGO, MARIA ENEYDA P. K. FIDALGO, AND JOÃO SALVADOR FURTADO, Instituto de Botânica, São Paulo, Estado de São Paulo, Brasil.

BIBLIOGRAPHY

Fidalgo, O. and M. E. P. K. Fidalgo. 1958. A new *Fistulina* from Brazil. Mycologia **50**: 145–146.

ISOLATION OF NOCARDIA ASTEROIDES FROM SOILS [1]

From a total of 102 soil samples, by means of paraffin baiting, 48 isolates of *Nocardia asteroides* and 16 other species of *Nocardia* were isolated. Criteria used for the identification of *N. asteroides* isolates were acid-fastness, colonial morphology and color, nitrate reduction and behavior on gelatin, casein, and starch. They were all acid-fast, displayed characteristic colonial morphology and color, produced nitrite from nitrate, were nonproteolytic on gelatin and casein, and nondiastastic on starch. The remaining 16 isolates were not identified to species but were representative of "soft nocardias" belonging to the morphological Group II described by McClung (Lloydia **12**: 137, 1949).

The soils in this study, which were collected from just below the surface using a flamed trowel and stored in sterile plastic bags, were from widely separated geographical areas: 41 were from desert soils in the area of Tucson, Arizona, 19 from West Virginia and Virginia (Webster and Buckingham Counties, respectively), and 40 from Ink's State Park, Burnet, Texas. Most of the soils from Arizona were sandy, but some from Mt. Lemmon were organic. The water content of the Arizona soils varied from 1–15%. The samples from West Virginia and Virginia were loam soils and contained 8–30% water. Those from Texas varied widely in composition and contained 3–35% water.

The isolations were made by a modification of the method of Gordon and Hagan (Jour. Infect. Diseases **59**: 200, 1936) as follows: A small amount of each soil sample was suspended in 5 ml of sterile basal medium without a carbon source ($NaNO_3$, 2 g; K_2HPO_4 (anhydrous), 0.8 g;

[1] The author gratefully acknowledges the support of this work by research grant No. E-2075 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Public Health Service.

$MgSO_4 \cdot 7H_2O$, 0.5 g; $FeCl_3$, 10 mg; $MnCl_2 \cdot 4H_4O$, 8 mg; $ZnSO_4 \cdot H_2O$, 2 mg; water to make 1 liter, pH 7.2). A paraffin-coated rod was prepared for each by inserting a 16 cm long glass stirring rod through a cotton plug into a 125 × 15 mm test tube containing about 4 g paraffin ("Tissuemat" Fisher mp 52–54° C). The paraffin was sterilized at 121° C for 15 min in an autoclave, after which the rods were carefully raised above the melted paraffin and allowed to cool. The paraffined rods were then transferred to the tubes of inoculated basal medium so that they projected about ½ inch into the liquid. These were then incubated at 37° C for two weeks or until growth was visible on the paraffin at the surface of the liquid. The rods were then removed and bits of the growth carefully transferred aseptically to a tube of melted and cooled nutrient agar with 1% glucose or glycerol. The inoculum was divided as finely as possible with the needle, thoroughly mixed, and one loopful transferred to a second tube of the melted cooled agar. The two tubes were then poured into sterile petri dishes and incubated until colonies appeared. Actinomycete colonies were spotted when young under the low power of a microscope and streaked onto plates of modified Jensen's medium (McClung, N. M., Trans. Kansas Acad. Sci. **54**: 218, 1951). Well isolated colonies from the streaks were then transferred to tubes of Bennett's medium (yeast extract, 1 g; beef extract, 1 g; N A Amine A, 2 g; glucose, 10 g; agar, 20 g; dist. H_2O to make 1 liter, pH 7.2), for further study. This method was found superior to earlier attempts which were made by streaking the paraffined rods directly onto the Jensen's medium.

Each isolate was then tested for paraffin utilization by the method described by McClung (Mycologia **47**: 424, 1955).

No correlation could be established between the number of isolates and geographical area, composition, or water content of the soil samples.

This study adequately demonstrated that *N. asteroides* can be isolated by this means from a variety of soils.

Results are summarized in the following table:

Location	No. of samples	Number of isolates	
		N. asteroides	*Nocardia* sp.
Arizona	41	20	3
W. Va. & Va.	21	16	9
Texas	40	12	4
Totals	102	48	16

The author is grateful for the technical assistance of Edna O. Thompson during this investigation.—NORVEL M. MCCLUNG, Department of Bacteriology, University of Georgia, Athens, Georgia.

THE GROWTH OF A FUNGUS IN INK

Many persons on the University of Missouri campus know that I am forever interested in locating fungi growing in, or on, unusual substrates. This means that I am occasionally flooded with dirty coffee cups with a blue-green mold growing on the unwashed residue—or with dishes of food forgotten in a refrigerator over vacation periods. It also means that truly unusual occurrences are brought to me from time to time. Such is the case with the occurrence that I wish to report in this note.

One of our graduate students filled his pen with a hypodermic syringe and then placed the syringe in a beaker of distilled water so that the syringe would not dry out and become stuck. (It now occurs to me that this is a strange procedure, although I did not interrogate him about it at the time.) At any rate, the student noticed several dozen fuzzy pellets in the inky water approximately a week later. He was able to establish that the pellets were fungal—no doubt because he had taken mycology from me a couple of years previously—and hence he brought the beaker to me.

It appeared that the fungus was subsisting entirely on the ink in the water, assuming, of course, that the water was clean. This seemed a novel occurrence to me. A survey of the literature revealed only one short paper concerned with deterioration of ink by fungi. Conover and Stevens (**1942**) discussed the discoloration of ink on soap wrappers by a *Penicillium* sp. I am not aware of any other reference to the growth of fungi on, or in, ink.

It was my impression that ink should be a relatively unfavorable nutritional substrate for the growth of a fungus. The question was, then, on what components of ink might a fungus subsist? The particular ink involved (Skrip, Permanent, No. 24, Blue-black) was a product of the W. A. Sheaffer Pen Company of Fort Madison, Iowa. Inquiry to the Sheaffer Company as to the composition of this ink brought prompt, complete answers to my queries from Mr. Robert S. Casey, Chief Chemist of the company. Mr. Casey graciously sent me several reprints discussing various aspects of ink manufacture, composition, testing and use. Nothing in these reprints referred to fungal deterioration of ink, but

Mr. Casey told me in his letter that the growth of molds and other microorganisms in inks is a troublesome problem and that it is necessary to add preservatives—usually phenol, thymol and other phenolic substances—to alleviate this spoilage.

The organism in the beaker of dilute ink was readily isolated in pure culture and grew on all of the several routine media on which it was seeded. It was not particularly surprising that it was a *Penicillium* sp. By using Raper and Thom (**1949**) it was readily established that the organism belonged in the *Monoverticillata* section. It apparently belonged in the *Ramigena* series because of the irregularly branched conidiophores. The organism did not grow restrictedly on Czapek's agar; the conidia were sub-globose, thick-walled, smooth and were produced in divergent chains. These characteristics placed the organism closer to *Penicillium waksmani* Zaleski than to any of the other members of the *Ramigena* series. However, it differed from *P. waksmani* in that (1) it was faster growing, (2) it did not present a strongly wrinkled and buckled colony, although some wrinkling was apparent, and (3) the conidia were smooth-walled. In view of the close resemblance between *P. waksmani* and *P. corylophilum* Dierckx (Raper and Thom, **1942**, p. 247), it appeared that this species found growing in ink was either (1) somewhere between *P. waksmani* and *P. corylophilum* with characteristics of both species or (2) a strongly monoverticillate strain of *P. corylophilum*.

In order to ascertain that the organism in question could grow on ink, solutions and solid media containing only the same commercially-obtained, autoclaved ink, distilled water and agar were made in concentrations of 5%, 10%, 25% and 50% ink. The fungus grew luxuriantly on both 5% and 10% ink agar, but would not grow on the ink agars of higher concentration nor on water agar used as a control. The organism grew well on the surface of sterile 5% ink solution—and later in various concentrations below 5% ink—but would not grow in higher concentrations of ink solution nor in sterile distilled water used as a control.

Casey (**1951**) indicated U.S.P. tannic acid as being the chief organic constituent of the type of ink in question. Gallic acid, in much less proportion, ferrous sulfate and soluble blue were also present and could play a part in the growth of the organism. Phenol and hydrochloric acid, further constituents of the ink, could hardly be considered as nutritional factors. Mr. Casey told me in our correspondence that he felt that the tannic acid was the principal component attacked by microorganisms. Consequently, sterile solutions of 5%, 20% and 50% U.S.P.

tannic acid were made and seeded with the organism. Solid media of these concentrations of tannic acid, plus agar, were also prepared, but these preparations never gelated regardless of the method of preparation; the plates were seeded anyway. Tannic acid did not support the growth of the organism in any of the concentrations tested.

In order to test whether the organism found in the original inky water had a particular metabolic specificity for ink in comparison to other related organisms, various species of *Penicillium* and *Aspergillus* were seeded onto 5% and 10% ink agars. Seven species of *Penicillium* (*P. notatum, P. camemberti, P. stipitatum, P. italicum, P. roquefortii, P. chrysogenum,* and *P. tardum*), four species of *Aspergillus* (*A. niger, A. fumigatus, A. oryzae* and *A. fonsecacus*), and seven as yet unidentified isolates of either *Penicillium* or *Aspergillus* which happened to be in my collection, were used for this purpose. All of the organisms grew on both 5% and 10% ink agar with the exception of *P. italicum*. The *P. italicum* in question had been isolated from deteriorating oranges and consequently might be expected to be somewhat specific in its growth requirements. The other seventeen isolates grew in varying degrees of robustness and at varying rates of speed on the ink agar, but there was no question about the growth of any of them.

On the basis of the above described cursory tests, it appears that the *Penicillium* sp. originally found in the inky water is not peculiar in its metabolic characteristics. It probably could have been any one of dozens of other species of *Penicillium* or *Aspergillus*—or, no doubt, other microorganisms as well. Ink appears to be just another of the multitude of substrates on which we know fungi to subsist. When the concentration of phenol and other inhibiting components of the ink is sufficiently high, as it is in commercial ink, little or no microbial action takes place. However, when ink is diluted, as it was in the beaker of distilled water and in the low media of ink concentration, the inhibitory action is lost and the ink becomes just another organic substrate for fungi.—JOHN E. PETERSON, Department of Botany, University of Missouri, Columbia.

LITERATURE CITED

Casey, R. S. 1951. Ink, writing. Encyclopedia of Chemical Technology **7**: 870–877.

Conover, R. A., and N. E. Stevens. 1942. A *Penicillium* "disease" of ink. Trans. Ill. St. Acad. Sci. **35**: 59.

Raper, K. B., and C. Thom. 1949. A manual of the penicillia. Williams & Wilkins, Baltimore. ix + 875 p.

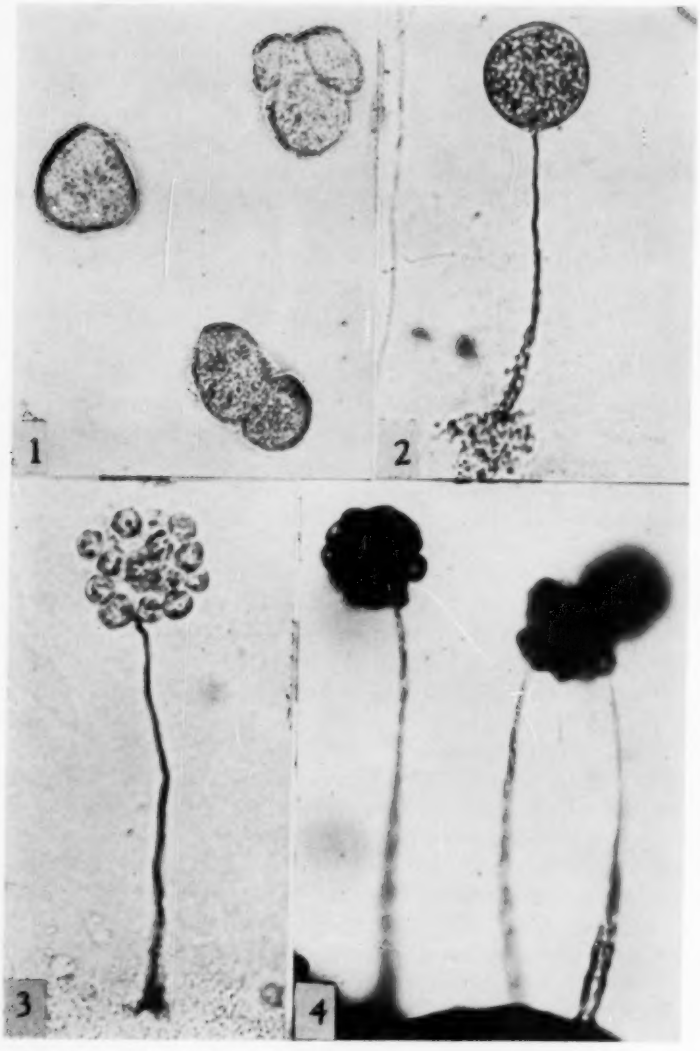
ECHINOSTELIUM MINUTUM

Recently, Alexopoulos (**1960**) published an excellent account of the culture and morphology of this minute myxomycete, which appears widespread, but because of its small size is infrequently collected. By coincidence we have been engaged in a similar study of the same organism. Since our results confirm those of Alexopoulos in almost every detail, we are publishing only this short note.

In our recent efforts to isolate cellular slime molds from dead plant material we observed the tiny sporangia of *E. minutum* De Bary on bits of old milkweed pods from Ardsley, New York, and fragments of dead mullein plants from near Westerly, Rhode Island, and New Hope, Pennsylvania, all collected in January of 1960 and plated out on cornmeal agar. Previously, this slime mold had been collected primarily on the bark of trees. When we isolated it on Difco cornmeal-dextrose agar with 0.1% yeast extract along with an encapsulated yeast and a bacterium from its natural substrate, the myxomycete grew well and fruited in a few days. Consequently, a number of other food organisms and agars were tested, and good growth and fruiting were obtained by growing the myxomycete on *Escherichia coli* alone or on *E. coli* and the encapsulated yeast on 0.1% dextrose-0.05% yeast extract agar. The best fruiting was obtained on *Phoma conidiogena*—which we have also found to be an excellent food source for two new cellular slime molds (Olive and Stoianovitch, **1960**)—contaminated with a bacterium.

Our cultures of this myxomycete are of the white variety, and we have not observed the pink-spored form mentioned in the literature. The fruiting bodies (FIGS. 2–4) are mostly 150–335 μ tall and the sporangia 31–94 μ in diameter. As Alexopoulos and others have noted there is a scanty but distinct capillitium arising from a disclike structure at the base of the sporangium. The thin peridial covering disappears before the sporangium matures. The number of spores in a sporangium varies from about a dozen to two hundred or more. The spores are larger than those previously described in the literature; they measure 9–13 μ in diameter. Small thickenings on the walls appear to gelatinize and enlarge during spore germination, which may occur within half an hour after transfer to fresh agar or a drop of water. A single amoeboid cell comes from the germinating spore.

Agar cultures of *E. minutum* show many small amoebae, microcysts and, after a few days, the unique microplasmodia (FIG. 1). These are never in the form of a network, but resemble large amoebae as they migrate over the agar surface. The microplasmodia fruit by producing a single sporangium as described by Alexopoulos.



FIGS. 1–4. *Echinostelium minutum*. FIG. 1. Microplasmodia, × 175. FIG. 2. Immature sporangium, × 400. FIG. 3. Mature sporangium in wet mount, × 400. FIG. 4. Sporangia on *Phoma* culture, × 400.

When the small amoebae that appear abundantly in young cultures are placed in a drop of water, many become converted into swimming cells within 15 minutes. Contrary to the report of Alexopoulos, who found the zoospores of his cultures to be biflagellate, these are, with few exceptions, uniflagellate.

A number of single-spore isolates was made. A little *E. coli* was mixed with the contents of a sporangium, and when the spores were separated with a micromanipulator and transferred to fresh plates, they were already inoculated with the food source. Of 33 single-spore cultures that survived, all sporulated, some within as short a period as one week. *E. minutum* is homothallic (if not amictic). The species is an excellent one for class demonstration. Because of its simplicity, ease of culture in the laboratory, and the existence of natural variants, it should be a very satisfactory myxomycete for genetic study.—LINDSAY S. OLIVE, Department of Botany, Columbia University, New York, N. Y.

LITERATURE CITED

Alexopoulos, C. J. 1960. Morphology and laboratory cultivation of *Echinostelium minutum*. Am. Jour. Bot. **47**: 37–43.

Olive, L. S., and Carmen Stoianovitch. 1960. Two new members of the Acrasiales. Bull. Torrey Bot. Club **87**: 1–20.

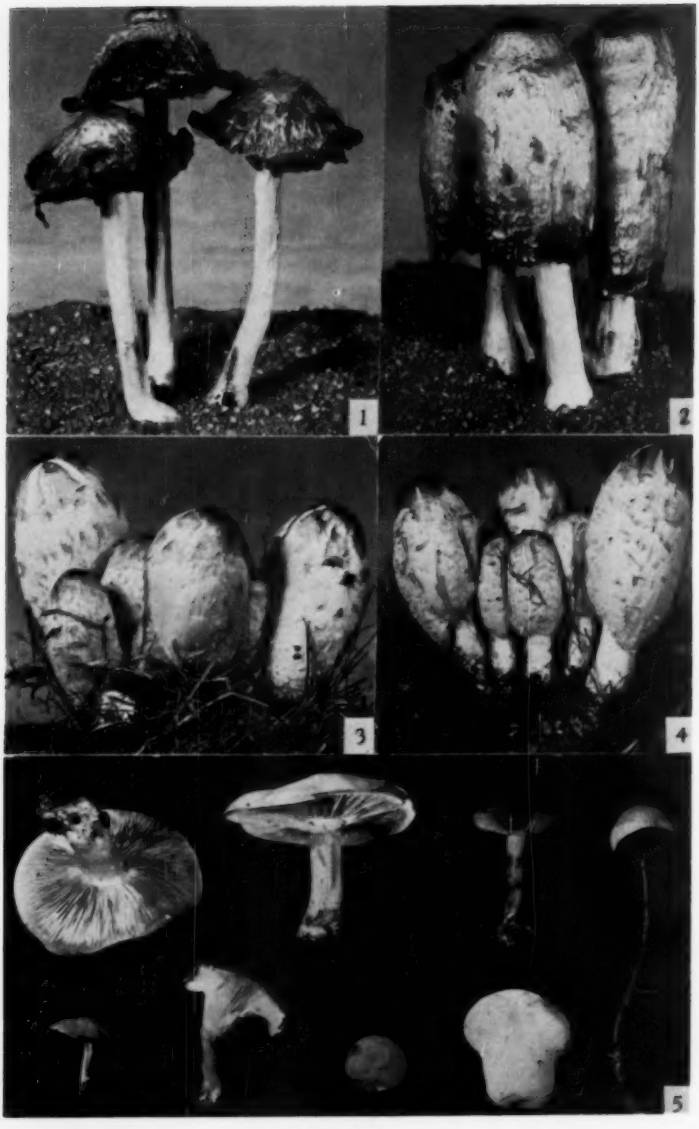
FREEZE-DRYING OF MACROFUNGI FOR DISPLAY

Interest shown in some of the author's display specimens of basidiomycete sporophores has prompted the publication of this brief article.

Mushrooms and the like have long been simply dried for storage as herbarium specimens. To speed up the drying process, various amounts of heat have been applied. The resulting specimens may bear little resemblance to the originals in size, shape and color. The internal structures are often so deformed as to be of little value in taxonomic studies.

Mercié (**1948**) was one of the first to use the technique of freeze-drying the intact fruiting body for permanent preservation of shape, structure and color. Stadelmann (**1959**) described a method of freeze-drying involving drying in a vacuum at 10° C for three weeks followed by storage in a museum jar. Completely covering the surface of the specimen with varnish provided a specimen that could be handled with ease. The present method is considerably faster, and makes use of apparatus available in many laboratories today.

The specimens illustrated (FIGS. 1–4) were brought in from the field



FIGS. 1–5.

in August 1959 and immediately placed in a cold room at −30° C. The following morning the frozen specimens were carefully placed in the primary drying chamber of an Edwards Model 3PS freeze-dryer and the apparatus turned on.

Sublimation of water from the specimen took place at a vacuum of 5–10 μ Hg with a cold trap temperature of −50° C and was essentially complete in 2–4 days.

At the end of the drying period, the specimens were removed from the drying apparatus and gently but thoroughly sprayed with several coats of clear acrylic plastic (Crystal Clear Acrylic No. DA1695, Aerosol Division, Dupli-color Products Inc., 2440 South Michigan Ave., Chicago 16, Ill.). The clear plastic, in pressurized cans, was particularly convenient to use.

Coating the specimen with plastic served to provide rigidity to the dry brittle specimens and to retard the uptake of water where the specimen was hygroscopic or the atmosphere humid. Stiffening wires may be inserted in the sporophores before freezing.

The specimens of *Coprinus comatus* shown (FIGS. 1–4) were stored under a simple dust cover in the open laboratory for 7 months before being photographed. The specimens of other basidiomycetous genera (FIG. 5) were freeze-dried and stored in a desiccator for over three years without apparent change.

Any apparatus satisfying the requirements of a good freeze-dryer can be used. The VirTis freeze-dryer is particularly useful because the drying chamber can be partially immersed in a dry-ice freezing bath whilst drying is taking place. While this will increase the drying period required, shrinking of the specimen is minimized and the specimen may be kept frozen in those cases where evaporative cooling is not sufficient to maintain this state.

This method of preserving whole sporophores is superior to heat drying. Shrinking is minimized, and color and other features remain essentially unchanged. Portions of the original habitat may be dried with the sporophores intact (FIGS. 3, 4). Specimens so prepared are particularly useful in the preparation of dioramas showing fungi, etc., in their natural habitats—the fine details displayed are unquestionably authentic!

FIGS. 1–4. *Coprinus comatus*. FIGS. 1, 2. Sporophores freeze-dried and arranged in dry soil (× 0.3 and × 0.4). FIGS. 3, 4. Young sporophores freeze-dried in original block of turf (× 0.4 and × 0.3). FIG. 5. Freeze-dried sporophores of various basidiomycetes (× 0.5).

The author wishes to thank Mr. A. S. Lutzko for assistance in preparing the illustrations.—R. H. HASKINS, National Research Council, Prairie Regional Laboratory, Saskatoon, Sask., Canada. (N.R.C. No. 6295.)

LITERATURE CITED

Mercié, C. L. 1948. Préparation des collections végétales par dessiccation sous vide (Nouvelles observations). Bull. Soc. Bot. France **95**: 38–43.

Stadelmann, Edward J. 1959. The use of Mercié's method of freeze drying for the preparation of fungi for demonstration. Proc. IX Internat. Bot. Congress, Montreal *2*: 375–376.

ANNOUNCEMENT

The herbaria of The New York Botanical Garden as a result of extensive renovation of the Museum Building housing the herbaria have been closed since June of 1960. Although initial schedules pre-supposed the completion of renovation operations within 120 days, it remains unlikely that the Garden's herbarium collections will be accessible to either Garden staff or visitors until July of this year.

During the period of the herbaria closure, more than a hundred requests for loans of herbarium material have been received at the Garden. That the specimens of a considerable number of these requests may no longer be needed is suggested by the cancellation of quite a few earlier made applications for loans.

In view of the probability that the need of many of the still outstanding requests has expired, we request all of our correspondents to submit a renewal of requests for loans, to become effective in July—or as soon thereafter as conditions permit. We hope that our colleagues throughout the world will be understanding of this interruption in the normal course of collaboration.

BASSETT MAGUIRE,
Head Curator.
April 21, 1961

REVIEWS

ILLUSTRATED GENERA OF RUST FUNGI, by George B. Cummins. ii + 131 p., *355 figs.* Burgess Publishing Company, Minneapolis, 1959. Price, $4.50.

This manual by one of the most able uredinologists gives in an easily accessible form keys and descriptions of the rust fungi of the world. The descriptions are chiefly a recapitulation of the key characters and in this way the reader's attention is focused upon those characters by which the genera are separated. All spore states present in each genus are illustrated. Those of the telial state are excellent camera-lucida drawings and photographs. Those of the other states consist mostly of clear schematic sketches, which by their simplicity and repetition reinforce the descriptions. The drawings and photographs reflect the author's wide knowledge of the rust fungi and his skill as an artist. Typographical errors are few, but the following words were misspelled: *U. longimucronata* p. 21, *M. flueggeae* p. 53, *D. woodii* p. 108 (legend), and *Calidion* Syd. p. 123. For some reason, too, authorities for the scientific names are given in the text and for the legends in the introduction, but not for those accompanying the descriptions although many of the species illustrated are not mentioned elsewhere.

In a rather brief introduction the author defines the spore forms and life cycles of the rust fungi. In his treatment he follows the concepts first proposed by Arthur. The spore forms as here defined depend not only on the morphology of the spores but also on their association with other spore forms or their mode of germination. Users of the manual must keep this difference in mind when consulting the older literature.

The description of each genus and the name of the type species are supplemented by some additional comment, but these notes are brief and could have been profitably expanded for several genera. For instance, in following recent practice of treating *Thekopsora* and *Calyptospora* as synonyms of *Pucciniastrum* his reasons for doing so would be welcome, as Pady (Can. Jour. Res. **9**: 458–485. 1933) presents evidence for their retention as separate genera. Moreover, no illustration is included of the type species, *Pucciniastrum epilobii*, a very common rust.

While a partial list of synonyms is adequate for the manual, the omission of some synonyms from the list and lack of reference to others

in the generic treatments lessen the usefulness of the manual as a quick reference. The treatment of *Corbulospora* under *Miyagia* and of *Bubakia* under *Phakopsora* is excellent but *Edythea* is not discussed under *Desmella* nor is the literature reference (Thirumalachar, M. J., and G. B. Cummins. Mycologia **40**: 417–422, 1948) included. *Phragmotelium* is commented upon under *Phragmidium* but not listed in synonymy. The monotypic genus *Mesopsora* is nowhere mentioned, yet the species is commented upon as *Melampsora hypericorum*.

This manual is a streamlined revision of Dietel's treatment (Die natürlichen Pflanzenfamilien, Band 6, 1928). It is undoubtedly more useful to the casual user, but less satisfying to a serious student with limited access to the world literature than the older classic.—I. L. CONNERS.

FUNGAL DISEASES OF ANIMALS, by G. C. Ainsworth and P. K. C. Austwick. 148 p., 8 half-tone plates, 4 color plates, 1 text-fig. Commonwealth Agricultural Bureaux, Farnham Royal, Bucks., England. Price, 20 s.

In this first comprehensive textbook on mycoses of animals, Ainsworth and Austwick have made a notable and useful contribution to veterinary medicine.

Their critical and judicial review of veterinary literature through 1957 reflects their thorough professional knowledge of mycology and their own wide experience with mycoses of animals. The fungal diseases of domestic animals have been neglected in veterinary research and in textbooks and this little book of 148 pages with 8 good black and white plates and 4 excellent and beautifully reproduced color plates now provides the teacher and the practicing veterinarian with an authoritative and practically useful text and reference book.

In 17 chapters the authors describe 17 mycoses and their etiologic agents, ergotism and the toxicoses caused by ingestion of mouldy fodder or grain. In the first chapter, "Aspergillosis," the authors are faced with the recurrent problem in evaluating the literature of veterinary mycology, *viz.*, the validity of reported etiology. They list 5 species of *Aspergillus* as pathogens and suggest that additional species alleged to have been etiologic agents in case reports may have been contaminants or erroneus identifications. Under "Avian Aspergillosis" the authors systematically list or discuss: disease name and synonyms, etiologic species, hosts, geographical distribution, symptomatology (acute and chronic), pathology (according to systems or organs), contributing fac-

tors, mycology of the fungus, epidemiology of the disease and its treatment and control. A similar plan is followed for "Mammalian Aspergillosis" and there is a section on experimental aspergillosis.

So far as it is applicable, a similar outline is followed in the presentation of candidiasis (regrettably the old and erroneous name "moniliasis" is used, although the presently accepted name of the fungus, *Candida albicans*, is adopted), cryptococcosis, epizootic lymphagitis, sporotrichosis, histoplasmosis, North American blastomycosis, coccidioidomycosis, haplomycosis, rhinosporidiosis, mucormycosis, bovine mycotic abortion, mycotic mastitis, actinomycosis, nocardiosis, streptothricosis (another unfortunate name for which as yet there is no satisfactory substitute) and ringworm. The list of toxin-producing fungi includes 20 species and a few toxic higher plants are mentioned briefly.

Although there was a selection of papers, the bibliography occupies 31 pages. The double-column 5-page subject index is adequate and well prepared with cross references.

Space allotted to each mycosis varies considerably. Chapter 1 on "Aspergillosis" occupies 13 pages, and Chapter 15 on "Ringworm" fills 16 pages, while the subject of actinomycosis (once predominant, perhaps, in veterinary mycology) is presented in 4 pages. These disparities are justified by the relative frequency of different mycoses, the limited geographic distribution of some, the degree of therapeutic control, or the need for stimulation of interest in an important but poorly known disease.

Probably the emphasis on aspergillosis, mucormycosis, mycotic abortion and ringworm reflects the authors' interest in these mycoses, in the study of which they have made recent and important contributions. The discussions of streptothricosis and "matted wool" are also illuminated by critical first-hand studies of these economically important mycoses.

In sections on epidemiology the absence of contagion in systemic mycoses and the importance of environmental sources of infection are emphasized. Recently acquired information about the ecology and saprophytic occurrence of such pathogens as *Aspergillus fumigatus*, *Histoplasma capsulatum*, *Coccidioides immitis* and *Cryptococcus neoformans* is reviewed. A useful table of fungus species, hosts and geographic distributions of dermatophytoses and a practical key to species of dermatophytes are included.

Few serious errors are apparent. However, in discussing the histopathology of cryptococcal mastitis on page 19 the authors note correctly the characteristic minimal tissue reaction in cryptococcosis and then review, without questioning it, the erroneous interpretation by Innes

et al. that tissues in mastitis were "dissolved." This interpretation by Innes revived an erroneous concept, long discredited, of a lytic action. The particular udder tissues upon which Innes based his interpretation were infected also by bacteria and, further, he failed to recognize properly the importance in these tissues of pressure exerted by the physical displacement of tissue by the growth of *Cryptococcus*. The report by Rector and Rector (p. 37) of coccidioidomycosis in the Townsend mole is probably erroneous. The association of *Actinomyces bovis* and fistulous withers of horses (p. 65) also is probably erroneous and the microorganism isolated may have been an anaerobic diphtheroid.

Readers unfamiliar with mycology will find that "Fungal Diseases of Animals" is not an elementary textbook of mycology with discussions of technics, or detailed and fully illustrated descriptions of the pathogens. The authors have written for the person with some knowledge of the fungi. The book is an excellent reference text, critical and adequate in its review of the literature of medical and veterinary mycology, authoritative in its discussions of the mycoses and their etiologic agents and stimulating in its presentation of the principles and problems of veterinary mycology.—C. W. EMMONS.

PERSOONIA. A MYCOLOGICAL JOURNAL. Editors, H. J. Lam, M. A. Donk, and R. A. Maas Geesteranus. Vol. 1, part 1, viii + 171 p., *illus.* Rijksherbarium, Nonnensteeg 1, Leiden, "1959" (Rec. Feb. 8, 1960); part 2, 130 p., 1960. Subscription price per volume (about 500 pp.), for institutes and societies, 25 Dutch guilders; for private subscribers, 20 Dutch Guilders; available on exchange.

A new mycological journal is always welcome; the first number of *Persoonia* has set an admirable standard for future issues, and earns a place with the best in its field. The journal supersedes *Fungus* and the earlier *Mededelingen van de Nederlandse Mycologische Vereniging* as the scientific publication of the Netherlands Mycological Society, but unlike these will be published in cooperation with the Rijksherbarium. Part 1 presents a historical preface (by Lam), three reviews of current books (Maas Geesteranus), and articles on *Plochmopeltis* (von Arx), *Sarcosoma, Actiniceps,* a new family of Sphaeriales, and a new *Septobasidium* (Boedijn), Cyphellaceae (Donk), *Hydnellum* and other stipitate Hydnaceae (Maas Geesteranus), *Geastrum* (Palmer), and a *Galerina* (Reijnders). One article is in German and one in French, the rest being in English. Part 2 is wholly devoted to a nomenclatural

article on Polyporaceae (Donk). Except in being restricted to mycology (including, apparently, lichenology) and to work from the Rijksherbarium, the new journal shows considerable similarity to the greatly lamented *Farlowia;* mycologists will hope to see it maintain equal distinction, and achieve a much longer life.—D. P. ROGERS.

LES CHAMPIGNONS HALLUCINOGÈNES DU MEXIQUE, ÉTUDES ETHNOLOGIQUES, TAXINOMIQUES, BIOLOGIQUES, PHYSIOLOGIQUES ET CHIMIQUES, by Roger Heim and R. Gordon Wasson, with several collaborators. *322* p. *37* pls. (*17* colored), 83 figs. (11 colored), 3 maps. Extrait des Archives du Muséum National d'Histoire Naturelle, Sér. 7, Tome VI. Paris, 1958. About $65.00.

This imposing quarto volume is essentially an *opus secundum* which complements the preceding monumental publication of the Wassons' by presenting the work of another partnership in a detailed and thoroughgoing study of the sacred mushrooms of the middle Americas in about all the remaining aspects.

Wasson's contribution is the first three chapters. The first deals with the early sources of information concerning these unusual agarics, as found in the accounts by the Spanish chroniclers of the 16th and 17th centuries; another discusses the mushrooms in Central American art and archaeology. The second chapter gives an account of his and Mrs. Wasson's trips to Mexico and their participation, probably the first by white people, in the rites, ceremonies and feasts concerned with these fungi, including the consumption of, at first two, and later three more, of the hallucinogenic species, and records their experiences and sensations, all in great detail.

The remainder of the book is the more technical contributions of Heim and his collaborators. Three chapters contain thoroughgoing and well-illustrated descriptions and the taxonomy of most of the 11 species of *Psilocybe* (two of them new) and one species each of *Stropharia* and *Conocybe,* the embryology and development of the carpophores of five species, and cultural studies of most of the species. Some mycological spice is contained in footnotes to the taxonomic section, and the disagreements with, and criticism of, American agaricologists provide opportunity for subsequent interesting rejoinders. There is a chapter on psilocybine and psilocine, their occurrence in the Mexican species, their production in culture, the structural formulae and chemistry, their pharmacology, toxicity to animals and effects. The final chapter discusses

the psychic effects of psilocybine and presents psychophysiological and clinical studies.

The volume is well-illustrated, both as to quantity and quality. The color plates are upon a glossy stock and therefore do not have the pleasing softness or mellowness of the plates of the Wassons' volumes but they are satisfactorily attractive. All but three are reproductions of water-color drawings, which, while not as artistic as some we have seen, are nevertheless adequate and complete with the numerous examples. The plates from color photographs are very good. Fourteen of the colored plates or figures are of fungi, all but one of the agarics under consideration. The half-tones are excellent, from photographs. Of the 73 figures in black and white, 42 pertain to the taxonomic portion of the book—32 of them illustrating microscopic details and 10, gross morphology. Altogether, about everything that could be desired in the way of illustration of the varied arguments and discussions has been accomplished in a clear and precise manner.

The paper is very good, the type is large and pleasing, and it reads easily, not only because of these features but also because of the plain and simple styles of the two authors and the other contributors. The editing appears to be good; at least, this reviewer found very few typographical errors.

The hallucinogenic fungi are not one of the present passions of this reviewer, but this work is interesting from all points of view, is definitely readable and is a valuable contribution, especially as completing within the limits of reason a rather vast and much ramified study initiated and stimulated by, as Heim states it, two New York ethnologists.—WALTER H. SNELL.

MANUSCRIPT

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